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# **Factors affecting maternal provisioning to the pre-natal environment**

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**Submitted for the degree of Doctor of Philosophy  
University of Edinburgh 2013**



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## Lay Summary

Many animals provision their offspring prior to birth by allocating resources to them. In mammals resources are transferred through the placenta, in birds resources are transferred into the yolk. These resources are known as maternal effects as they impact on the offspring post birth and are not just a product of a mother's genetics but of her environment too. Maternal effects can be highly beneficial to the offspring, for example, mothers can transfer protection (maternal antibodies) to pathogens she has come into contact with, which can provide offspring with protection prior to their immune system being fully developed. However, there is variation in transfer of maternal antibodies between and within individuals. Production of offspring/ eggs can be costly to a mother, therefore uneven allocation of resources to different eggs or clutches can occur to increase a mother's chance of producing successful offspring. A number of factors can affect the level of allocation eggs and clutches receive; male traits, position within a clutch, breeding attempt and offspring sex, along with other environmental effects.

In this thesis, I investigate how parent traits impact on maternal allocation within and across reproductive events. I use a laboratory based experiment using Chinese painted quail (*Coturnix chinensis*), a small game bird, to examine the role that mothers and her mates play in clutch traits including the level of antibodies individual eggs receive. Maternal effects are an important source of resources for offspring. Exploring in detail the factors which can affect them will lead to a better understanding of female's ability to allocate to offspring differently depending on the environment she is in and the impact this has for health and fitness across a family.



## Abstract

Maternal effects are important mechanisms by which mothers' may influence the phenotype of their offspring. Females may vary in the resources they can provide during offspring development and understanding the factors responsible for this variation is key to understanding offspring success- in early life as well as later life. Differential allocation has been reported to occur, however how it impacts on offspring and mother's future reproduction still remains unclear. This is also true for maternally transferred substances like maternally transferred immunity. Contributions to date have been limited to snapshots in time, mean level of transfer and/or limited information regarding other maternal traits. For my thesis, I aim to further the understanding of maternal allocation effects and explore the transfer of maternal antibodies over an immune response of a mother, across multiple breeding attempts and accounting for embryo, maternal and paternal traits. Furthermore, I determine the effect of key male traits on general egg traits along with maternal antibodies. I examine this at the individual level using Chinese painted quail (*Coturnix chinensis*) who are prolific layers and sexually dimorphic.

To date the majority of differential allocation studies have not necessarily addressed the assumptions of differential allocation theory. In Chapter 2 of this thesis I attempt to address some of these assumptions and explore the impact of male characteristics across a number of clutches and find separate effects of initial pairing and subsequent pairings. I found that mothers can create, by differential allocation, clutches of varying size but egg components (egg mass) appears to be largely influenced by initial clutch pairing and not by paternal traits. Furthermore, the effect on egg mass appears to be a secondary effect mediated by females adjusting their condition based on their initial pairing. I demonstrate that unlike general clutch traits (clutch size, egg mass) maternal antibodies are not affected by male characteristics (Chapter 3) carry-over effects of egg size means antibody levels may be influenced throughout life by early experiences. However, maternal immune response may be detrimentally linked to viability of offspring. Whereas maternally transferred antibodies appear to have no relationship with maternal or paternal traits, oocyte yolk antibodies during development were found to correlate with female antibodies up to 48hr prior to lay. In Chapter 4, I examine a neglected area regarding maternal effect- exploring variation between female in their transfer of antibodies. Individual females were highly consistent in the relative level of specific blood antibodies transferred to eggs across different phases of their immune response, across challenge types (bacterial and viral) and that some females consistently transfer significantly more than others. The relative level of circulating antibody transferred was independent of the individual's overall strength of antibody response and related to the female's body condition (while the individual's own antibody responses were not). We found no evidence for any trade-offs between the amount transferred and overall reproductive investment in this chapter. In Chapter 5, I discuss the wider implications of my findings and suggest future research directions.

## **Declaration**

I declare that I have composed this thesis, under guidance of my supervisor. I conducted all experimental work on birds involved and collected the data, with help as detailed below, and all analyses are my own. This work has not been submitted for any other degree or professional qualification.

**Chapters 2, 3 and 4** laboratory skills (antibody extraction from yolks and ELISA methods) were outlined by Dr Vincent Staszewski.

**Chapters 2 and 3** are based on the experiment I carried out in the 2012 and all experimental work, data collection and lab work is my own.

**Chapter 4** includes data collected by myself and help from Dr Katherine Herborn in 2010/ 2011, the analysis is my own.

*(Christina Marie Coakley)*

## Acknowledgments

This PhD would not have been possible without the support and guidance of many people. Firstly, I would like to thank my supervisor Emma Cunningham, who has kept me on the straight and narrow over the past four years; her scientific contributions have been invaluable. She has been patient, has given me constructive comments and warm encouragement, and overall has been a fantastic role model who I have learnt a lot from. I am also thankful to my second supervisor Loeske Kruuk who gave me helpful feedback during manuscript writing. I am deeply grateful to Vincent Staszewski, my unofficial supervisor who helped me enormously in the early stages of my PhD. Not only did he help me with technical methods in the laboratory but he was great fun to work with. I would like to offer my special thanks to Katherine Herborn, who supported me in one of my experiments. Her contributions to the workload and the company she gave was super-human. I am indebted to her for her contribution on those long sampling days, along with keeping me sane, and for her help and encouragement with getting to grips with R. A technical thank you goes to Gidona Goodman for her veterinary input throughout the course of my thesis. She has really been the go-to person for any quail dramas that have occurred and I have really appreciated her help. I also thank NERC for funding my study over the last few years and the quail who were used in this project.

I thank my fellow PhD class of 2009: Laura Appleby, Tom Black, Hanna Granroth-Wilding, Jess Hedge and Steph Smith, for the huge amount of support they have all given me (in their very different ways), and for all the fun we have had in the last four years. You guys have been there every step of the way and I couldn't have asked for a better group of friends to help me through this thesis! I would also like to thank the lovely people from my department, who have always been willing to give advice and encouragement when needed, particular thanks goes to – Hanna Granroth-Wilding, Jenny Garbutt, Sue Lewis, Tom Little, Sarah Matthey and Lynsey McInnes for reading chapters. I also thank Melanie Narish for allowing me to use a piece of her art work in my thesis.

My final thanks go to all my family, in particular my brother and sister, for being superb siblings and helping me with proof reading! To my wonderful husband Alex, who has helped me keep things in perspective in my write up, has proof read documents and has always been there for me. Finally, last but not least, thanks go to my parents Jacqueline Barnett and Stephen Barnett, for supporting me throughout my life and never giving up on me; you really have got me to where I am today.

*Στην πεθερά μου*

# CHAPTER 1

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## Introduction

### 1.1 Overview

A fundamental aim of biology is to understand why individuals vary in their health and fitness. An individual's genotype will obviously play a major role however recent research has emphasised the importance of the developmental environment in determining how traits are expressed throughout an individual's life. Mothers, in particular, play a key role in shaping early developmental conditions for offspring and it has recently become apparent that mothers can vary widely in the pre-natal environment they provide (Mousseau and Fox, 1998b). Recent studies have demonstrated that the nutritional, hormonal and immunological environment created by the mother for a developing embryo can be influenced by a diverse range of factors, such as choice of partner, levels of stress, and exposure to disease. Such maternal effects are now known to impact on offspring throughout their life (Mousseau and Fox, 1998b, Myatt, 2006). The exact mechanisms governing these effects remain unclear. Furthermore, despite a large number of studies now demonstrating a female's embryonic investment may be altered under particular conditions, there is little information on how long these effects persist, the consequences for such changes for current and subsequent offspring or the consequences for maternal survival and health. Understanding why differences in early provisioning may occur, what mechanisms are involved and what the consequences are for different offspring produced across a female's lifetime is now key to understanding the impact of maternal effects on individual health and fitness.

## **1.2 Provisioning of offspring**

### **1.2.1 Provisioning and maternal effects**

Many species produce offspring that are highly vulnerable after birth. The success of these offspring can depend on many external factors, such as environmental conditions, food availability, levels of infection and social environment. Conversely, the effect of these factors on offspring can be dramatically changed by the care provided by the parents and post-natal care strategies have been widely studied (Royle et al., 2012). In a range of taxa, including birds, mammals, fish, insects, reptiles and amphibians, provisioning of offspring can also occur prior to birth. Mothers can provide a range of nutritional, immunological or hormonal resources which can either provide direct benefits to the offspring and/or allow the offspring to concentrate their own resources on processes such as growth and development (Grindstaff et al., 2003). Females in a population may vary widely in the level of resources they transfer to their offspring. Part of this variation may have a genetic basis; egg size for example, is a key trait in determining the level of resource a mother allocates to her young and has a strong genetic component (Christians, 2002). However, egg size may also reflect the environmental conditions a female has faced during her lifetime (Christians, 2002). Maternal effects, whereby the overall phenotype of a mother can directly affect the phenotype of her offspring, independently of the offspring's own phenotype, can account for a significant amount of variation in offspring traits (Wilson et al., 2005). It has therefore been suggested that they should be explored independently of other environmental or genetic effects (Mousseau and Fox, 1998a). Recent work has focused on exploring the transfer of specific components of the embryonic environment that are thought to be beneficial, or even crucial, for offspring development and success: in birds, for example, higher levels of maternal antibodies in eggs can impart greater protection to offspring from disease before their own immune system is fully developed (Rose and Long, 1971), higher androgen levels in eggs can be important in establishing social rank of chicks in the nest (Pilz et al., 2003), greater transfer of a range of nutrients can improve offspring survival to hatching (Wilson, 1997) and a higher level of transfer of antioxidants to the egg can induce greater antioxidant activity in chicks

after hatching, which have been known to prevent cell damage and can act as an immunostimulant (Blount et al., 2002). However, in some circumstances, maternal effects can have a negative impact on offspring development. For example, mothers with elevated levels of stress hormones can transfer higher levels of corticosteroids or glucocorticoids to offspring, which can result in decreased birth weight (Newnham et al., 1999) or learning ability (Braastad, 1998). There may therefore be an optimal amount of allocation for any particular component for any individual.

Maternal effects generally have a substantial impact on the early phases of offspring development, but maternal provisioning during pre-birth stages can also have longer-lasting effects. For example, the effect of vaccinating mothers can be detected in the immune response of their offspring in adulthood, long after maternal antibodies have degraded (Reid et al., 2006). Given that maternal effects can shape an individual's phenotype across their different life stages, maternal effects are of fundamental evolutionary importance. By combining with the influence of offspring genotype, maternal effects play a key role in generating a range of individual phenotypes in a population on which selection may act. Consequently, maternal effects can contribute to evolutionary change in the population by both accelerating or dampening genetic effects (Räsänen and Kruuk, 2007).

### **1.2.2 Maternal effects, life-history and differential allocation theory**

Maternal condition can be a key aspect determining the level of transfer of resource that can be transferred by a mother to offspring. However, many species reproduce multiple times throughout their life and parents must therefore balance how much they invest in any given breeding attempt in relation to the impact this may have on their own survival and their ability to invest in future mating opportunities. Life-history theory predicts that parents should alter their reproductive investment in relation to both the likely pay-off from the current breeding attempt and the expected pay-off that might be obtained from future reproductive events (Stearns, 1992). Natural and sexual selection is therefore expected to have acted on allocation

strategies that maximise a female's reproductive success across her life. Prenatal allocation has been predicted to vary in relation to the value of offspring being produced, with greater investment expected in more valuable offspring to maximize the return on the amount invested across a female's lifetime. Offspring may vary in their value to the mother either because offspring vary in their chance of survival and/or in their likely success in reproduction. This may vary with their paternity (as fathers may differ in resources which they provide, or the genetic traits they confer to their offspring); their sex (as sons and daughters often differ in their requirements or likely success in a given set of circumstances) or their position in any family hierarchy (as siblings in different positions may differ in their prospects from other siblings). There has been particular interest in how allocation patterns may covary with male traits as these responses may not only have a direct impact on offspring fitness but alter the strength of sexual selection on male characteristics.

### **1.2.3 Differential allocation in response to different males**

Under the differential allocation hypothesis (Burley 1986), females are predicted to adjust the amount of resources they allocate to offspring in response to male traits that relate to the value of a breeding attempt. Males may vary both in the direct material benefits they provide and/or the indirect genetic benefits they may pass on to their offspring; if these benefits are sufficient to compensate for any cost that females may incur by increasing their level of investment then differential allocation should occur. In Burley's classic study she experimentally manipulated male attractiveness to females by giving males either a red or green leg band (females prefer more red males in this species) (Burley and Coopersmith, 1987)) and found that females altered their reproductive allocation in response to this experimentally manipulated cue (Burley, 1986, Burley, 1988). Other studies have since gone on to show similar patterns of resource allocation in response to manipulation of male traits that are favoured by females (Table 1.1).



**Table 1.1** Studies exploring differential allocation on reproductive traits in avian species. \* indicates pairings with two males of different attractiveness for individual females, RC= reproductive compensation, ↑= increased allocation to preferred partner and ↓= decreased allocation to less preferred partner.

Species	Allocation explored	Differential allocation		Publication
		Traits affected	Direction	
Barn swallow	Clutch size, brood size, nestling success	brood size, more clutches	↑	De Iope et al. 1993 ( <i>Evolution</i> )
Barn swallow	Clutch size, number of clutches	number of clutch	↑	Kose et al. 1999 ( <i>Anim. Behav.</i> )
Blue tit	Clutch size	clutch size	RC	Delhey et al. 2007 ( <i>Behav. Ecol.</i> )
Blue tit	Clutch size, testosterone and androstenedione	testosterone & androstenedione	↑	Kingma et al. 2009 ( <i>Behav. Ecol.</i> )
Blue-footed booby	Egg mass	egg size	↑	Dentressangle et al. 2008 ( <i>Behav. Ecol.</i> )
Blue-footed booby	Egg Mass	Egg mass	↓	Velando et al. 2006 ( <i>Oecologia</i> )
C.p quail	Egg mass, clutch size	egg size	↑	Uller 2005 ( <i>Behav. Ecol. Socio.</i> )
Canary	Egg Mass	egg size	↑	Leitner et al. 2006 ( <i>Ethology</i> )
Canary	Egg mass, clutch size, androgen	androgen	↑	Tanvez 2004 ( <i>Gen. Comp. Endo.</i> )
Chicken	Clutch size	clutch size	↑	Forkman & Corr 1996 ( <i>Appl. Anim. Behav.</i> )
Gouldian finch *	Clutch size, egg mass	clutch size, egg mass	↑	Pryke & Griffith 2009 ( <i>Science</i> )
House sparrow	Clutch size, food provisioning, chick weight, number fledged	-	-	Nakagawa et al. 2007 ( <i>Oecologia</i> )
House wren	Clutch size	larger clutch, more sons	↑	Dubois et al. 2006 ( <i>Proc. R. Soc.</i> )
Magpie	Clutch size, incubation start date	clutch size	↓	Soler et al. 2001 ( <i>Behav. Ecol.</i> )
Mallard	Egg mass	egg mass (2nd yr mother)	RC	Bluhm et al. 2004a ( <i>Anim. Behav.</i> )
Mallard	Number hatched, survival	number hatched, survival	↑	Bluhm et al. 2004b ( <i>Anim. Behav.</i> )
Mallard *	Egg Vol	egg size	↑	Cunningham & Russell 2000 ( <i>Nature</i> )
Mallard *	Egg mass, lysozyme, carotenoid	egg size and lysozymes	↑	Giraudeau et al. 2011 ( <i>Proc. R. Soc.</i> )
Peafowl	Egg Mass, yolk testosterone	egg size, testosterone	↑	Loyau et al. 2007 ( <i>Behav. Ecol.</i> )
Peafowl	Clutch size	clutch size	↑	Petrie and Williams 1993 ( <i>Proc. R. Soc.</i> )
Pied flycatcher	Lay date, clutch size, egg size	egg size	↑	Morales 2006 ( <i>J. Ornithol.</i> )
Pied flycatcher	Egg mass, lay date, clutch size	egg mass	↑	Osorno et al. 2006 ( <i>J. Ornithol.</i> )
Spotless starling	Clutch size, egg mass, yolk androgen	clutch size	↑	Lopez-Rull & Gill 2009 ( <i>J. Avian Biol.</i> )
Zebra finch	Egg mass, clutch size	clutch size, mass	↑	Arct et al. 2010 ( <i>Bio. Lett.</i> )
Zebra finch	Size of-clutch, number hatched & final brood	number hatched	↑	Burley 1986 ( <i>Amer. Naturalist</i> )
Zebra finch	Egg mass	egg size	↑	Gilbert et al. 2006 ( <i>Proc. R. Soc.</i> )
Zebra finch	Clutch size	-	-	Gorman et al. 2005 ( <i>J. Avian Biol.</i> )
Zebra finch *	Clutch size, egg mass, egg proteins & lipid	clutch size	↑	Balzer et al. 1998 ( <i>Behaviour</i> )
Zebra finch *	Egg Vol, carotenoids	egg vol, carotenoids	RC	Bolund et al. 2009 ( <i>Proc. R. Soc.</i> )
Zebra finch *	Egg Mass	egg size	↑	Holveck et al. 2010 ( <i>Proc. R. Soc.</i> )
Zebra finch *	Egg mass, clutch size, yolk androgen	egg mass	carry-over	Rutstein et al. 2004 ( <i>Anim. Behav.</i> )

Differential allocation is usually considered to describe an increase in allocation for a preferred partner, but in long lived species it may also be beneficial to decrease allocation to less preferred partners if there is a high chance of securing a better partner in the future and resource allocation constrains female breeding in the following year. In a study of the blue-footed booby (*Sula nebouxii*) females produced smaller eggs when paired to males that had been experimentally manipulated to be less attractive compared to females paired with either controls or males manipulated to be more attractive. This implies that females reduced rather than increased reproductive allocation when faced with attractive or unattractive males in this study (Velando et al., 2006). Both these forms of DA have a similar effect however, in that they result in a positive correlation between a male trait value and levels of allocation.

However, an alternative pattern of allocation can also occur where females actively increase allocation for offspring of less preferred partners. This phenomenon is known as reproductive compensation (RC). The term was originally used to explain the tendency for parents to invest in maintaining a given family size when offspring are lost (and hence why deleterious recessive alleles that lead to such losses may persist more than expected) (Overall et al. 2002), however it has more recently been used to describe how females might allocate resources to compensate for situations where females have no choice but to pair with a less preferred partner; reproductive compensation is used to reduce any negative effects of this mating on her reproductive success (Gowaty et al., 2007). This can lead to different consequences as RC results in a negative correlation between a male trait value and levels of allocation. Positive differential allocation to preferred male therefore reinforces sexual selection on a trait and may overestimate genetic contributions if not accounted for (Räsänen and Kruuk, 2007). Reproductive compensation may reduce the selective pressure on preferred traits and potentially mask the importance of genetic contributions.

The majority of studies have found positive correlations between male trait values and levels of investment to be more prevalent than reproductive compensation (Table

1.1) and the first comparative studies have suggested a theoretical prediction that females will on average invest more in mating with a preferred partner compared to less preferred partners and that allocation in the opposite direction (RC) is likely to be rare (Horvathova et al., 2011). However, there are cases where both patterns have been reported in the same species; in zebra finches for example, six different studies have found females lay larger eggs or more eggs for preferred males (Arct et al., 2010, Balzer and Williams, 1998, Burley, 1986, Gil et al., 1999, Gilbert et al., 2006, Holveck and Riebel, 2010) while one has found a pattern consistent with reproductive compensation with females producing larger egg volumes with less attractive mates (Bolund et al., 2009); in the mallard three studies have found females laid larger eggs when paired to preferred males (Bluhm and Gowaty, 2004b, Cunningham and Russell, 2000, Giraudeau et al., 2011) but one found patterns consistent with reproductive compensation in some older females that hadn't mated previously (Bluhm and Gowaty, 2004a); and in the blue tit- two studies have found feeding rate to be greater for offspring from preferred mates (Johnsen et al., 2005, Kingma et al., 2009) but one study found clutch size to decrease when with preferred males consistent with reproductive compensation (Delhey et al., 2007).

There are a number of possibilities that may explain why findings may vary between these studies: for example, maternal age may change an individual's physiology or the pay-offs obtainable from different allocation choices; Previous reproductive experience may determine future reproductive allocation; maternal condition may constrain a female's ability to differentially allocate and resource availability may also influence level of resources available to reproduction. Some authors have also examined how allocation varies between different individuals when they lay a single clutch and others have looked at allocation by the same female across several clutches (Table 1.1). Both are important in examining allocation decisions and their consequences, but to directly test the differential allocation theory, namely that individual females should vary their allocation between males of different preference, the latter approach is required. Studies are now required to investigate what underlying differences in breeding conditions can induce these very different reproductive strategies in different populations of the same species.

Very few studies have looked at differential allocation across a significant proportion of a breeding lifespan or the consequences of these allocation decisions on offspring or female success. Without an appreciation of how differential allocation might affect a female's offspring over her lifetime reproductive output, it is difficult to draw conclusions about whether this phenomenon is adaptive. Many studies that find evidence for differential allocation assume that it is. However, only a few studies to date illustrate the consequences of allocation for maternal productivity (Bluhm and Gowaty, 2004b) or offspring traits that are likely to be associated with survival (Cunningham and Russell, 2000, Gilbert et al., 2006).

Female allocation decisions have generally been assumed to be under female control and that resources should be distributed in a way that will maximize their reproductive success. However, differential allocation decisions also have to be viewed in relation to the interests of the other parties with a genetic interest in any given breeding attempt. There may be conflicts of interest over how resources are allocated between males and females and between parents and offspring and allocation decisions are likely to reflect elements of this conflict and may or may not be at the optimal level for any of the parties involved. If females are likely to produce offspring of mixed paternity over her lifetime, males may attempt to maximise their short term fecundity when his eggs are being fertilised and induce females to invest in reproduction to a greater extent than may be optimal for female fitness. For example, male seminal fluids in *Drosophila melanogaster* are known to increase a females egg output in the short term; this benefits the male who has most recently mated but females incur associated costs that decreases their lifespan (Chapman et al., 1995). These differences in optimal allocation can lead to conflict and depress the reproductive rate of both parties involved (Eldakar et al., 2009). Conflict may also occur between parent and offspring as any individual offspring is likely to want a greater share of resources than a mother may want to give, as she may benefit more from partitioning resources between other offspring and her own condition to facilitate future breeding events. The expression of paternally and maternally derived genes in offspring, for example can function antagonistically whereby offspring

genes function to gain increased resources from the mother while maternal genes may function to resist offspring demand (Haig, 2000). Such conflicts may further impact on patterns of allocation and the consequences they have for the success of different offspring.

### **1.3 Differential allocation of specific components of the embryonic environment**

Many studies of differential allocation have focussed on overall allocation decisions as reflected by changes in egg mass or clutch size. However, how these decisions affect the relative allocation of different egg components remains unclear. If, for example, egg size is increased, are all egg components increased in equal proportion or do some components contribute more to these differences than others?

Furthermore, might individual egg components vary between breeding attempts irrespective of any changes in egg size or number. Different components of the embryonic environment such as nutrients, antioxidants, antibodies, carotenoids, corticosteroids and androgens can have a major impact for offspring both early in development and later in life (Mousseau and Fox, 1998b). For example, experimentally injected androgen in eggs has been found to increase bouts of aggression and dominance status in Black-legged Kittiwakes (*Rissa tridactyla*) (Muller et al., 2012) and prenatal corticosteroid (stress hormone) exposure has been found to affect foraging behaviour in Japanese quail (*Coturnix japonica*) (Boogert et al., 2013). It is now clear that choice of partner may also influence these individual components in a number of different ways (Gil et al., 1999, Saino et al., 2002a). One major component that can have a major impact on offspring success is the transfer of maternal antibodies.

### **1.4 Maternal antibodies**

When an animal encounters a pathogen in the environment, physical barriers are a very simple yet effective part of the immune system: for example the skin and mucous membranes present in the digestive and respiratory tracts can block many microorganisms from infecting an individual, and serve as an important first line of

defense. However when an antigen (toxic or foreign substance) gets past these barriers, other parts of the immune system are required to prevent damage.

The avian immune system is similar to that of the mammalian immune system, in that antigenic stimulation instigates an immune response that requires cellular components, such as macrophages, B-lymphocytes and T lymphocytes (Sharma 1991). Macrophages process the antigen and present it to the lymphocytes. B-lymphocytes are the mediators of the humoral immune system, when signalled they change to plasma cells and produce specific antibodies, which are present in the body fluids. These antibodies (proteins) bind to the antigen and prevent it from entering cells, and signal for its removal by macrophages. T lymphocytes are part of the cell-mediated immune system, and their function is mediated by lymphokines.

Antibodies are a very small component of the immune system, although they are a part of the acquired immune system, which has been shown to transfer directly to chick via the egg. Antibodies are part of the acquired immune response due to their response to specific pathogens. Antibodies bind to a specific antigen of the pathogen, preventing it from entering a cell. They also signal removal of the antigen by macrophages. Acquired immunity can be further subdivided into two groups, passive and active. Passive immunity is when the immune system of the individual being challenged with an antigen is not stimulated. Instead, immunity (like antibodies) has been transferred to them; for example maternal antibodies, which are present in the egg prior to birth. With active immunity, the immune system of the individual being challenged is stimulated, causing an immune response. On the first encounter it is slow and mild (primary response); non cellular by the B lymphocytes and cellular by the T lymphocytes. Active immunity leads to memory of a pathogen, resulting in a faster and better response in subsequent exposures (secondary response).

Antibodies play an important role in defence against many kinds of pathogen and the antibody response in a female who is reproductively active and producing offspring can be transferred directly to the developing embryo prior to birth. Such immune protection is known as passive immunity as the immune system of the individual receiving the antibodies is not being stimulated itself by an antigen. The maternal

effect of transferring immunity to offspring is widespread throughout different animal groups including mammals, birds and invertebrates (Boulinier and Staszewski, 2008). In mammals, these substances can be transferred directly from the mother prenatally through the placenta and post-natally through the colostrum (first milk) and continued milk (Glezen, 2003). For example, in humans maternally transferred antibodies have been linked to early life protection from parasites such as malaria (Fried et al., 1998). In birds, most substances (including maternal antibodies) are transferred to the yolk when the egg is in oogenesis (Rose and Long, 1971) before sperm reach the egg and fertilisation takes place and prior to other egg components such as the albumen and shell being added. These antibodies will be a mix of general protection molecules that are always present but may also include more specific molecules raised in response to a specific set of pathogens that have been encountered by a mother. They will therefore often represent the pathogen environment the offspring are likely to face when they are born. Paternal antibody levels do not seem to correlate with egg and chick antibody levels, and authors have suggested there is no vertical transfer of immunity from the father (Gasparini et al., 2002).

#### **1.4.1 Benefit of maternal antibodies**

In birds and mammals, the immune system only develops after birth, so offspring are at high risk from pathogens in the period immediately after birth. Young animals focus a considerable amount of energy into growth, such that at this stage the impact of mounting an immune response may incur high costs. Maternal antibodies can reduce the cost of immune defence (Rose and Long, 1971, Staszewski et al., 2007b). In addition, equipped with maternal antibodies, offspring are prepared with specific defence for the pathogen environment they are being born into. This transfer of specific antibodies can play an important role in the survival of populations during epidemics, and surviving early infection can lead to long lasting protection for individuals (Navarini et al., 2010). It is believed that this is because energy that would be used to mount an immune response is conserved, and therefore energy is directed toward development instead (Grindstaff et al., 2003). Offspring with

maternally transferred antibodies can show increased growth and earlier fledging compared to offspring without maternal antibodies (e.g. Grindstaff, 2008). Offspring survival has also been noted to be positively affected by maternal antibody transfer; Heller et al., 1990 for example, concluded that there was a correlation between hens' antibody titre and the percentage of the progeny that survive.

#### **1.4.2 Cost to the mother of maternal antibody transfer**

The immune response is generally thought to trade-off with other life history traits and have a substantial cost on both survival (Hanssen et al., 2004) and future reproduction (Ardia, 2005). When female birds are producing eggs, their circulating antibody levels increase compared to non-laying individuals (Klasing, 1998). This may occur due to the additional antibody requirement for the transfer of maternal antibodies to offspring while needing to maintain a certain level of circulating antibodies for their own immunity. In birds, much of the early literature from poultry science suggests the level of antibody transfer is invariant and that females consistently transfer approximately 20% of their circulating antibody level to each egg produced (Brambell, 1970). Due to the increased antibody production by mothers and transfer to eggs, antibody transfer is believed to be negatively related to female condition, with resource limitation (impacting on female condition) stated as a mediator of variation in maternal antibody transfer (Grindstaff et al., 2003). However, the main body of evidence to support this suggested trade-off between condition and maternally transferred immunity has found mixed results from positive correlations between maternal antibody transfer and female condition under both natural conditions (Hargitai et al., 2006) and supplemented food conditions (Pihlaja et al., 2006), to a negative relationship under supplemented food (Gasparini et al., 2007) and no effect under supplemental feeding (Grindstaff et al., 2005). This suggests that there is no direct/simple trade-off between immunity and condition and that, the relationship between the two is likely to depend on the environment which the individual is experiencing at the time and the pathogens and resources it encounters.



### **1.4.3 Variation in transfer (passive or active)**

Like other maternal effects, maternal antibody transfer was previously believed to be a passive process reflecting circulating levels of antibodies of the mother when the egg was produced (Kowalczyk et al., 1985). However, a large number of studies have now reported different levels of maternal antibodies in eggs consistent with variable levels of female transfer (Blount et al., 2002, Saino et al., 2002b, Muller et al., 2004, Grindstaff et al., 2005, Pihlaja et al., 2006). Moreover, a number of studies have put forward evidence for variation in maternal antibodies that may not reflect the mother's antibody (based on a limited number of maternal blood samples at various points throughout reproduction) (Saino et al., 2002b, Hargitai et al., 2006, Martyka et al., 2011), suggesting that females could actively vary the level of antibodies her offspring receive. While a considerable amount is known about how individuals generate their own immune response, far less is known about mechanisms underlying the transfer of this immune response from mother to offspring or, indeed, how they co-vary with a female's ability to generate an immune response in the first place.

### **1.4.4 Maternal allocation of antibodies (differential allocation)**

Whether active or passive, the transfer of large numbers of antibodies to offspring is likely to be an expensive process for the mother, with trade-offs against other life history traits such as reproduction or survival (Sheldon and Verhulst, 1996). Thus, females are expected to make decisions on allocating antibodies to offspring based on offspring value and her condition (see Section 1.2.2). Exploration of paternal traits on maternal antibody transfer has only been explored in a handful of studies (Saino et al., 2002b, Hargitai et al., 2006). While Saino et al. found evidence to support differential allocation theory on maternal antibody allocation, Hargitai et al. did not, and given the small amount of literature published to date, no clear patterns are visible. Variation in antibodies may occur via several different mechanisms: 1) female immune response may vary when paired to different males and this may passively be reflected in offspring produced, 2) females may actively transfer more

or less antibodies to offspring of a given male 3) females may vary their allocation in egg traits when paired to different males and antibody level or concentration varies as a result e.g. if females lay larger eggs do they produce more antibodies to keep the concentration level the same or the same level of antibodies but their concentration is therefore proportionately lower? To fully understand how males may influence female allocation of antibodies studies need to consider the level of antibodies present in the female when she is laying eggs and control for other forms of differential allocation that may be occurring

In the past it has been quite separate fields of research with separate bodies of literature that have investigated the roles of differential allocation and maternal antibodies on offspring success, and as yet, there is no overall understanding of how these allocation mechanisms interact.

## **1.5 Industry**

Due to the benefits seen in the early life of individuals with maternally transferred antibodies, this process has been harnessed as a vaccination strategy in the poultry industry (Wallach et al., 1992, Ziomko et al., 2005). A vaccine is used to induce an antibody response in the mother, and these antibodies are transferred to all the hen's chicks while she is responding to the vaccine, protecting them from the pathogen in early life (Goddard et al., 1994, for review see, Wallach et al., 1995). This type of prophylactic approach is thus economically favourable, and also allays concerns over drug use to control disease. For example, for control of *Eimeria* (the protozoan parasite responsible for coccidiosis in avian species), medicating individuals through feed was standard practice, but resistance is emerging (for review see, Chapman, 1984). Studies into vaccinations have directed the industry towards taking advantage of the mechanism of maternally transferred antibodies to prevent infection in the first place (Wallach et al., 1992, Ziomko et al., 2005). This process has also been used to produce vaccines collecting antibodies present in the eggs (Schade et al., 1991). Selective breeding on the basis of female immune responses have been considered as a means of maximising levels of immune protection in the poultry industry.

However, whether the mechanisms of higher levels of maternal substances in chicks are due to a greater transfer (of maternal antibodies or other resources), or greater circulating levels in the mother (for example antibody levels), is still undetermined (for review see, Boulinier and Staszewski, 2008).

## **1.6 Study system**

In this thesis, I investigate how females allocate resources in response to different partners under different pathogen environments created by vaccination over the major part of their reproductive lifetime. I examine these questions in a laboratory setting using domesticated Chinese painted quails (*Coturnix chinensis*) which is an ideal model system for these types of question. The Chinese painted quail is a small game bird in the Phasianidae family, found from India to South East Asia. Quail, like other egg producing species, produce an embryonic environment external to the mother in the form of an egg, containing everything that is required by the young bird from fertilisation until hatching and therefore exploration of maternal effects can be less invasive than in species with internal development. Quail are precocial so there are also no confounding effects of parental provisioning post birth and although socially monogamous, frequently mate switch between breeding attempts (Hoyo et al., 1992) so are likely to face allocation decisions at each breeding attempt. As in other avian species, females store sperm following copulation. Maximal sperm storage length in this species has been estimated to be 7 days  $\pm$  2.09<sub>SD</sub> (Cunningham et al. unpublished data). Wild type males and females are sexually dimorphic, with male plumage containing a black and white proportion on the neck area referred to as a badge. Different females use this cue to base decisions on how to allocate to their first clutch when mated to different males (Uller et al., 2005). Females produce large clutches (~ 9 eggs) over a number of days (Hoyo et al., 1992) and eggs hatch synchronously and can be artificially incubated with high hatching success. They are particularly useful for exploring life-history questions as they have a relatively short life span of one to three years, and adapt easily to captive conditions with their small size (40-85g), low aggression and reproductive success in captivity (Tsudzuki, 1994).

Quail also respond to vaccines produced for common diseases affecting poultry and other wild species and tools are already available to quantify antibody responses.

## **1.7      *Aims and outlines for thesis***

The overall aim of this thesis is to explore factors which affect prenatal provisioning levels within and between reproductive events and the implications this may have for parent and offspring success. I first look at overall differences in allocation in terms of egg size and egg number and then more specifically at maternal antibodies following different types of immune challenge by vaccination. In Chapter 2, I firstly investigate the impact of male characteristics on female allocation decisions across several different breeding attempts and examined the consequences of initial allocation decisions on subsequent decisions and the associated implications for offspring success. I then looked at the impact of these decisions on embryonic growth and viability. Finally, I also examine variation between females in their ability to allocate resources and its potential consequences on maternal condition. In Chapter 3, I ask whether antibody transfer can vary according to partner's traits within a clutch, explore the mechanisms by which this might occur and address the issue of differential transfer between offspring sexes by using a novel technique to measure egg antibody levels before and after incubation. In this chapter I also explore potential costs of maternally transferred antibodies and production of circulating plasma antibodies on female condition. In Chapter 4, I investigate between-individual variation in females' ability to transfer antibodies to their offspring. Furthermore, I determine how repeatable this is across different immune challenges and explore whether the ability to transfer antibodies relates to various female fitness traits. In Chapter 5, I discuss the broader implications of my findings, highlight outstanding questions and suggest future directions for research of these topics.

## CHAPTER 2

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### **The Impact of male and female traits on reproductive allocation**

#### **2.1 Introduction**

##### **2.1.1 Differential allocation (DA) theory**

Early environmental conditions can play a key role in determining offspring success. In most cases this environment is created by the mother who must allocate a range of resources to each of her offspring. Throughout life, females must therefore partition resources between their own self-maintenance and different reproductive events over their lifetime. For a female's reproductive success, her choice of partner can play a major role, both via direct material benefits he may provide and the genetic traits he passes on to her offspring (Andersson, 1994). Life history theory would therefore predict the relative costs and benefits of different allocation strategies to vary with different mating partners, and that plasticity in allocation decisions could potentially maximise maternal and offspring success (Mousseau and Fox, 1998b). The adjustment of reproductive allocation to offspring arising from mating with different males is known as differential allocation theory (Burley, 1986). Differential allocation (DA) theory was first demonstrated by Nancy Burley who showed that females allocate more resources to offspring of preferred mates in zebra finches (*Taeniopygia guttata*), and that this allocation could be manipulated experimentally by altering the perceived attractiveness of the partner. Differential allocation, whereby females invest more in a reproductive event with some males over others has subsequently been reported to occur in a range of species across taxa (Sheldon, 2000).

### 2.1.2 Potential benefits arising from initial mate choice

There are two broad types of benefit females can receive from their mate that could influence female allocation decisions— direct material benefits and /or indirect genetic benefits which impact on offspring fitness and therefore impact on female reproductive fitness indirectly. These may be signalled via male traits that can be assessed by females making allocation decisions. Direct benefits such as courtship gifts, access to resources on a high quality territory or even protection from harassment that allows females more time to feed, can potentially alter the overall level of resources a female has available to allocate to reproduction as well as the value of the reproductive attempt *per se*. These additional resources need to be controlled for when examining different levels of allocation for different males to assess whether females are actively targeting a greater share of resources to breeding attempts with a particular male. However, there are other direct benefits and indirect benefits that may simply alter the likely success of a given breeding attempt without making additional resources available – these might include direct benefits such as preferred males having nest sites more protected from predation, for example, or indirect genetic benefits for traits that influence offspring mating success or viability. There has been particular interest in DA in relation to any genetic benefits that males may confer to offspring as maternal effects may either mask or over estimate quantitative estimates of genetic contributions on offspring success (Cunningham and Russell, 2000, Gil et al., 1999, Sheldon, 2000) and have the potential to accelerate or dampen selection on parental traits (Räsänen and Kruuk, 2007). However, differences in allocation may also occur in response to male traits that indicate the likely value of breeding without any directional selection arising. For example, in waterfrogs (*Rana lessonae-Rana esculenta*), females actively invest less ( as they actively release fewer eggs) for unattractive hybrid mates which have been shown to produce less successful offspring (Reyer et al., 1999). Females may also adjust allocation to reproductive events differently due to how closely related a male is to her and therefore on the likely success of the potential offspring produced (Arct et al., 2010). In these situations DA may occur on the basis of female assessment of males – but no directional selection on any particular trait would occur.

Differential allocation is often assumed to be uni-directional, in that females are expected to increase investment when with high ranking males as females would benefit from a proportionately higher pay off overall. However, differential allocation may also be bidirectional or compensatory (Ratikainen and Kokko, 2010). For example, female blue-footed boobies (*Sula nebouxi*) invest less than control females when assigned to males with less preferred traits (duller blue legs which are used in sexual displays during courting). This may make sense in long-lived species that breed over many years and in which breeding allocation in one year impacts on the next. For example in common eider ducks (*Somateria mollissima*), females with experimentally increased clutches in one year had reduced fecundity the following year (Hanssen et al., 2005). Whether females invest more for more preferred males, or less for less preferred male, both lead a positive correlation between male trait values and allocation levels. However, the opposite pattern of allocation may also occur. Reproductive compensation occurs when females invest more in breeding attempts with less preferred males - females may compensate for being paired with a low “quality” (or less attractive) individual to balance the potential negative effects that less preferred males could have on offspring fitness which is likely to occur when mate choice is limited (Gowaty et al., 2007). The majority of studies have found female allocation to be positive in relation to preferred males and only a handful which found evidence for reproductive compensation. In keeping with this, Harris and Uller (2009) examined the conditions under which variation in mate traits should result in female allocation variation and concluded cases of reproductive compensation may be limited (occurring only when the relative impact of parental investment on offspring quality was low) and that increased investment when mating with preferred mates would be the most common optimal investment strategy for females

Differential allocation is assumed to benefit offspring, however the impact of changes in allocation on offspring traits that may be associated with survival or reproductive success have only been explored in a small number of studies (Cunningham 2000, Gilbert 2006). Additionally, a common trend in the literature is to explore the effects of differential allocation once hatching has occurred, while this

takes into account any affects of differential allocation post birth it ignores the affect of DA pre birth such as embryo viability. Only a few studies to date illustrate the consequences of allocation for maternal productivity and implications of DA offspring over multiple clutches (Bluhm and Gowaty, 2004b, Cunningham and Russell, 2000).

### **2.1.3 Mate traits and DA theory**

DA theory can be tested via exploration of male traits which females use as cues to assess male fitness and studies have aimed to explore these effects via experimental alteration of appearance or attractiveness. These types of experiments are an important tool in testing DA theory as the cues of fitness are manipulated but other characteristics of the individual remain unchanged. In zebra finches, “redness” is a signal that female zebra finches consider when selecting a mate. Burley’s classic studies manipulated perceived attractiveness of males altering male overall “redness” by addition of red or green leg rings (Burley, 1986, Burley, 1988). Experimental manipulation of sexually selected traits has been achieved in a number of species, for example, via elongation or shortening of tail feathers in barn swallows (Saino et al., 2002b) and manipulation of forehead badge size in pied flycatchers (Morales et al., 2006). However as with any manipulation, it is unclear if allocation would occur based on natural variation or if manipulation may potentially handicap an individual instead of increasing their attractiveness (for review see, Sheldon, 2000).

Other have focused on natural variation in male traits using female preferences directly and found female traits such as egg size (Cunningham and Russell, 2000) and clutch size (Petrie and Williams, 1993) vary on the basis of initial preferences; demonstrating differential allocation occurs within the range of natural variation in a population. DA theory has now been widely reported to occur across species in a wide range of species (Table 1.1) and replicated in different studies of the same species. However, it is not clear how many of these examples are truly differential allocation. Rutstein et al. (2005) highlight three assumptions of DA theory that are often not fully considered. Firstly, there should be evidence of differences in allocation between breeding attempts with different males by the same female,



second, individual should be flexible in their allocation response to environmental and social cues, and third, that trade-offs should exist between increasing allocation levels and future reproduction and/or survival (Rutstein et al., 2005). Based on these three points many studies do not meet these criteria. One of the main reasons is that many studies only explore allocation patterns over one reproductive event and focus on differences between females as opposed to within females (Table 1.1). This results in studies only looking at a snap-shot of a female's reproduction, whereas DA theory is implicitly based around the fact that the same females are predicted to alter their allocation when placed in different breeding situations. It is clear from observed life history trade-offs that reproductive events are not independent of each other and therefore, to investigate differential allocation multiple breeding attempts need to be explored. Some studies have controlled for this by using an experimental set-up where females are given either attractive or unattractive individuals and then give the opposite to determine if they change. By doing this individual's act as their own control and more importantly studies can explore the change in allocation between the two different male partners.

It is clear that a mother's allocation decisions to a given reproductive attempt are not an independent event. Previous experience may affect allocation decisions - not just in terms of any trade-off based on how much allocation has previously been used but also because previous experience might alter a female's perception of breeding conditions or her likely breeding success. For example, allocating females to different partners that vary in their attractiveness can alter a female's perception of her own attractiveness/dominance status (Collins, 1995) and this may impact onto subsequent reproductive attempts. These types of effect whereby previous investment in a given breeding attempt can influence subsequent levels of investment have been called "carry-over effects", while this term is often used to describe a left over effect of a previous treatment in experimental design (Ruxton and Colegrave, 2011) or effects across a seasons (Harrison et al., 2011) the term fits the occurrence of this phenomenon (Rutstein et al., 2004) and, therefore, when exploring DA across multiple clutches carry-over should also be explored to make sure this effect is either controlled for or are explored independently.

#### **2.1.4 Who can differentially allocate?**

Many variables may impact on the allocation to different reproductive attempts. Male traits may play an important role but additionally how flexible females can be to these cues is also of importance. A female's ability to change allocation in the face of different events (including the "quality" of the partner she is paired with), could be defined by a particular trait such as her condition (Heaney and Monaghan, 1996). For example, individuals of low condition may be less able to increase their allocation. One study shows some evidence for this, where egg mass in zebra finches was found to have a significant positive relationship with female condition, but only when paired with attractive males (Rutstein et al., 2005). And in mallards, females that initially laid large eggs altered their allocation levels to a much greater extent than females that initially laid smaller eggs (Cunningham and Russell 2000). Alternatively there may be genetic variation in plasticity which results in a selective advantage to being able to be plastic (for reproduction) as it may increase fitness, as seen with breeding date (Nager and Noordwijk, 1995). Life history theory predicts there are trade-offs when you allocate more resources to one event as less resources will be available for future reproductive events. With individuals who are of a low condition, one would predict this would be more prevalent as there are fewer resources to allocate, and so any change in allocation will have a big impact on subsequent allocation and therefore decisions are made at a higher stake. Exploring differential allocation therefore should also include the monitoring of traits related to female condition to pull apart these effects and how female condition could impact on differential allocation.

In this study I explore allocation decisions, addressing the assumptions of DA theory, of females over the major part of their reproductive life where they are paired to multiple males (four partners) in Chinese painted quail. In Chinese painted quail the badge size of the paired male has been found to influence the mass of their female's eggs (Uller et al., 2005) when females first paired with a male, suggesting that differential allocation can occur in this species. Furthermore, in this species a positive relationship between mating behaviours (time to copulation and number of copulations) and individual's badge size has also been found (Uller et al., 2005),

suggesting that badge size could be an honest secondary sexual characteristic reflecting male fitness. Despite this study, it remains unclear if differential allocation occurs across multiple breeding attempts (as hypothesised by DA theory) throughout their lives, or what the consequences of this are for mother and offspring. My first objective was to determine if male secondary sexual characteristics such as badge size or body size are taken into account in female reproductive decisions, and whether patterns arising in initial allocation decisions were consistent across the major part of a female's lifetime. The second part of the chapter then looks at the consequences of any differences in allocation on embryo growth and development. Finally, I explored whether all females respond in the same way or if female traits play a role in governing allocation decisions throughout their life.

## **2.2 Methods**

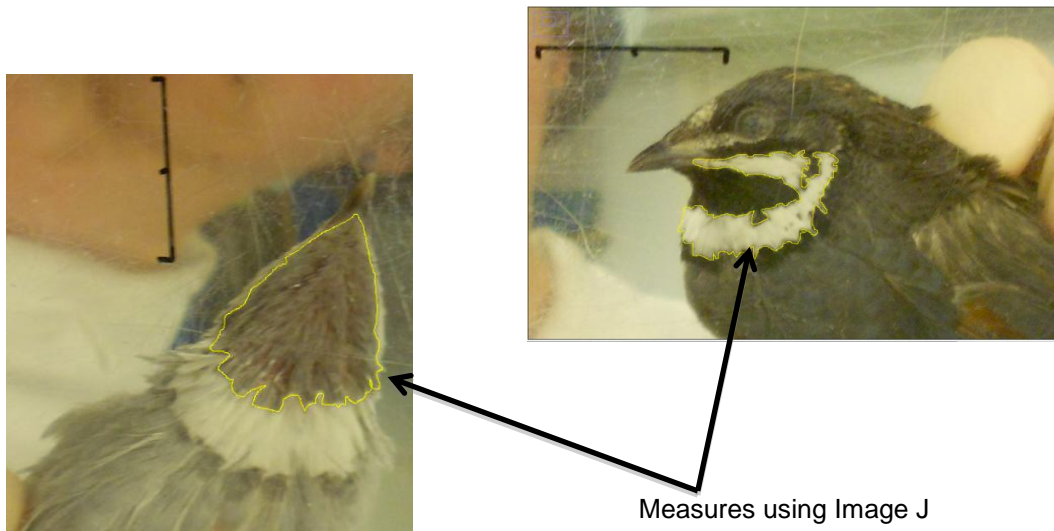
### **2.2.1 Study system: Establishing the maternal generation**

The study was conducted using a colony of Chinese painted quail (*Coturnix chinensis*), established at the University of Edinburgh. Chinese painted quail are small game birds in the Phasianidae family and are socially monogamous but frequently mate switch between breeding attempts (Hoyo et al., 1992). They have also been noted as being an ideal candidate for laboratory experiments due to their small size (40-85g), low aggression and reproductive success in captivity (Tsudzuki, 1994). To establish the parental generation, eggs were collected from multiple commercial breeders around the UK and sprayed with Ambicide™ (1% dilution) prior to incubation to prevent any transfer of common environmental pathogens into the colony. Eggs were incubated under standardised conditions (37-38 °C and 40-50% humidity rising to 70% prior to hatching) then brooded for 24 hours prior to transfer to communal cages. Chicks were fed *ad libitum* and kept in large (2,400 x 500 x 375 mm) mixed sex cages (14 birds per cage) until they reached sexual maturity. Cages consisted of wood shavings, multiple feeding stations, covered areas and sand baths to allow birds to follow their full repertoire of natural behaviour. A heat lamp was provided in one corner until 10 days post-hatching. Birds were ringed to allow individual identification at six weeks of age. Standard biosecurity measures to maintain a pathogen free flock were in place throughout the duration of colony establishment and the experiment.

### **2.2.2 Male morphological traits**

Chinese painted quail are sexually dimorphic; males have a distinct black and white patch on their throats (referred to as a “badge”). Badge size has been found to be an important trait affecting allocation decisions by different groups of females; a group of females paired to larger badged males were found to lay larger eggs than a group of females paired to smaller badged males (Uller *et al.*, 2005). In the wild, males of this species are also known to provide direct benefits to females via courtship feeding. However, whether individual females use this cue to alter allocation decisions when mated to different males across a series of clutches as predicted by

DA theory remains unclear. Measurements of overall badge size and the relative areas of black and white components of the badge were achieved by taking standardized photographs of each bird. Images of the badge were obtained by holding an individual against a clear piece of plastic in 2 positions; front facing so that the front of the badge was exposed and side facing to measure its full extent (Image 2.1). The total area of the badge and the relative areas of the black and white components were calculated using the software image J 1.43 (see Image 2.1). Replicate images of the badge were taken one week later. Measurements were repeatable over time (repeatability; front white ( $r= 0.94$ ), front black ( $r= 0.77$ ), side white ( $r= 0.82$ ) and side black ( $r=0.70$ ). No males moved from categories large to small or small to large between badge size measurements.



**Image 2.1** Yellow lines in images represent area measured (Image J software).

The mass of all males was measured to the nearest 0.1 grams every four days during the experimental period. Pectoral muscle and fat were assessed using BTO guidelines (Ringers' Manual BTO, Thetford); pectoral muscle was scored 0 if the sternum was sharp and muscle depressed; 1 if the sternum was still distinguishable but not sharp and 2 if the muscle was rounded over sternum. Fat levels were scored 0 if no fat was visible in tracheal pit, 1 if there was a trace of fat and 2 if the tracheal pit was obscured by fat. A skeletal morphometric was determined using the tarsus length; the right tarsus of each bird was measured to the base of the foot with an accuracy of 0.01 mm using electronic callipers. As this was an experimental set up with birds kept under standardized conditions, body condition index was calculated by mass / tarsus length<sup>3</sup>, though different measures of condition revealed qualitatively similar results (see, Galvan, 2010, for discussion).

### **2.2.3 Female morphological traits**

Female morphometrics including tarsus length, weight, muscle and fat scores were measured as detailed previously for males. A female's baseline egg size was established prior to the experiment by taking the weight, length and width of all eggs laid prior to housing with any given male and calculating the average weight and volume of an egg laid prior to pairing.

## **PART A**

### **2.2.4 Measuring allocation decisions in response to male traits**

Birds were housed throughout the experiment in their experimental pairings in breeding cages (800 x 500 x 375 mm). Each cage was lined with wood shavings, and contained a nest area for the female and a sand bath. Adult birds were maintained on a photoperiod of 16 h:8 h L:D and on a diet of Haith's finch seed, EMP, Prosecto Insectivorous and oystershell in a mix of: 20% protein, 2.5% calcium and 77.5% seed for the duration of the experiment. Total feed per cage was 35.8g based on 17.9g of feed per bird, which is 10% less than how much an adult laying female consumed *adlib* over a 24hour period (unpublished data). Biometrics of birds (total body mass (g), tarsus length (mm)) were measured and baseline blood samples were collected. A subgroup of birds were vaccinated with an avian vaccine or phosphate buffer solution (PBS) as part of another experiment; treatments were therefore equally balanced across experimental groups and treatment was included in all models to account for potential differences between groups.

Females were initially paired with a randomly allocated male. Each male was specified as either being of a large or small badge size. Pairs were left undisturbed to initiate and lay a full clutch of eggs. Once egg laying started, eggs were removed on a daily basis but replaced with an identical dummy egg in the same location as the egg was found to induce females into laying a natural clutch. At day 15, males were removed from their partners and the dummy clutch of eggs removed to induce females to lay a further clutch of eggs. Males were then allocated to another female's cage; females previously housed with large badge males were housed with small badge males and vice versa; this was repeated a further two times to collect four clutches of eggs from each female – two from pairings with large badged males and two from pairings with small badged males.

Eggs were measured using callipers and weighed to allow egg volume, clutch size and total number of eggs produced to be recorded for each pairing over the course of the experiment.

## **PART B**

### **2.2.5 Measuring the impact of allocation decisions on embryo viability**

Eggs were collected on a daily basis and their mass, length and breadth were recorded. Eggs were then directly transferred into incubators with automatic turners to be incubated under standard conditions (37-38 °C and 40-50% humidity). After 3 days, eggs were removed from the incubator and stored at minus 20°C until further analysis was conducted. Eggs were then carefully dissected and the presence or absence of embryos was recorded. All egg components were then weighed to record the weight of the embryo mass (if present), yolk mass and albumen mass (Image 2.2).



**Image 2.2** Photograph of dissected egg; embryo, yolk and albumen labelled.

Embryo characteristics and allocation levels may vary depending on the sex of the offspring being produced. To determine sex of the embryo DNA was extracted from the embryo using *DNeasy kit (Qiagen)* following the methodology provided by the kit. The samples were then sexed using the polymerase chain reaction (PCR) to amplify part of the W-linked avian CHD gene (CHD-W ) in females, and its non-W-linked homologue (CHD-Z) in both sexes, using PCR primers 2718R and 2550F (Fridolfsson & Ellegren 1999). Products were run on a 2% agarose gel and visualized with ethidium bromide and the presence of either one (CHD-Z; males) or two distinct bands (CHD-Z and CHD-W; females) were used to determine sex. All eggs that developed a visible embryo were successfully sexed (n= 100).



### **2.2.6 Statistical analyses**

Before conducting analyses, normality of residuals and homogeneity of variance were checked and transformations completed to achieve normality. To examine how allocation changes in response to male traits over a female's reproductive lifetime all data were analysed using linear mixed effect models (using the software "R"). For questions related to allocation to a female's first clutch, models included female identity as a random factor. For questions that explore differences in allocation across several clutches, female and male identities were included as random factors and clutch was nested by female ID. Female traits that may influence levels of reproductive allocation were explored by including female baseline condition index as covariates in all models. Egg mass, clutch size and embryo mass were also explored as covariates when not in models as dependent variables. A backward stepwise procedure was used such that non-significant terms were sequentially removed from the model. Male badge size was defined as large (area= 140.24-194.23 mm<sup>3</sup>) or small (area= 100.58-137.16 mm<sup>3</sup>). Male condition was also included as a covariate to determine their effect on allocation. No correlation was found between male condition and badge size (n= 20);  $r = 0.29$ ,  $p = 0.132$ , male mass and badge size;  $r = 0.02$ ,  $p = 0.378$ , or male tarsus length and badge size;  $r = -0.01$ ,  $p = 0.987$  (Pearson's correlation).

Binomial reproductive traits (such as viability or embryo sex) were analysed using the statistical modelling package "lmer" using the family- binomial, for all other models package "lme" was used (exploring dependent variables such as clutch size, egg mass and embryo mass). Factors affecting sex ratios of clutches were analysed using linear mixed effect models, binomial error and logit link function. In each analysis, the number of sons divided by the number of daughters plus sons was fitted as the response variable and the numbers of offspring sexed in the brood was included in the model to account for number of offspring. A G-test was performed to test sex ratio (sons/number sexed) difference from 0.5, using goodness of fit. Any non-significant terms from models presented in text are presented from when they were removed during model simplification.

**PART A The effect of male traits on female allocation decisions**

**2.2.6.1 Models examining initial allocation levels**

To determine the effect of male traits on a female's initial allocation decisions, the data was subset to first examine how females allocated resources to their first clutch produced with the first male paired with. Model 1 (Table 2.1) highlights all the main effects used to explore egg mass in first clutches. To determine the direction of any significant change in egg mass at first pairing with a male, baseline egg mass and egg volume was recorded for each female prior to pairing. A GLM was then performed to establish if there was any change between baseline egg mass and first clutch egg mass and if there was any interaction with male badge size (female ID was included as a random effect).

**2.2.6.2 Models examining allocation levels across a female's reproductive lifetime**

To explore whether females differentially allocated between breeding attempts with different males, the effect of male traits on the same dependent variables as Model 1 were explored across all four clutches that a female produced (two with small badged males and two with large badged males) (Model 3, Table 2.1). Again, the dependent variables; egg mass, viability, embryo sex, embryo mass and clutch size were examined and clutch order. A further model was run (Model 2, Table 2.1) which had two additional predictor variables, used to explore 'carry over' effects of which male a female was initially paired with: these were "Initial male" and "Paternal male". "Initial male" refers to the badge size of the male a female was originally paired with in the first clutch produced and "Paternal male" refers to the badge size of the male the female is paired with for a given clutch. Carry-over effects are often an effect which is to be avoided in experimental design to allow treatment effects to be examined without bias from previous experimental treatments, for example if individuals are to receive multiple treatments leaving sufficient time between them to avoid confusion of effects of different treatments (Ruxton and Colegrave, 2011). However in this study carry over effects were examined to determine if any effect of paternal traits during initial pairing continued on to subsequent breeding attempts.

Data was subset to only include clutches 2, 3 and 4 and models were run either including or excluding Paternal male (if Initial male traits were significant). Model 2 (Table 2.1) was used to determine the effect of Initial male on subsequent clutch allocation, the model was then run again to include Paternal male to determine if Paternal male explains egg mass better.

**Table 2.1** Models to determine male effects and female effects on reproductive traits.

Model	RESPONSE	EXPLANATORY	RANDOM	Data
1	Egg mass	Female baseline condition Clutch size Embryo sex Male badge size Male condition Male Mass	Female ID	Clutch 1
		<b>Interaction</b>		
		Female baseline condition*Clutch size Male badge size*Clutch size Male badge size*Embryo sex		
2	Egg mass	Female baseline condition Clutch size Embryo sex Initial Male badge size Initial Male condition Initial Male mass Paternal Male badge size Paternal Male condition Paternal Male mass	Female & Male ID	Clutches 2,3 and 4
		<b>Interaction</b>		
		Female baseline condition*Clutch size Initial Male badge size*Clutch size Initial Male badge size*Embryo sex Paternal Male badge size*Clutch size Paternal Male badge size*Embryo sex		
3	Egg mass	Female baseline condition Clutch size Embryo sex Male badge size Male condition Male mass	Female & Male ID	All Clutches
		<b>Interaction</b>		
		Female baseline condition*Clutch size Paternal Male badge size*Clutch size Paternal Male badge size*Embryo sex		

**PART B** *The effect of allocation decisions on embryo viability and growth*

*Models examining effects of allocation on embryo characteristics and viability*

Viability, embryo sex and embryo mass models were constructed in the same way as models in part A.

## 2.3 Results

The patterns of allocation over the course of 4 consecutive clutches were followed for 12 females who consistently produced eggs. Females were paired to two large badge males and two small badge males. The order in which they received males of different badge size was balanced across treatments; half the females were paired to large, small, large, small badge males and half were paired to small, large, small, large badge males.

### **PART A**

**Table 4.2** Linear mixed effects models exploring egg mass in initial allocation (Model A1), the impact of initial male on subsequent clutches (Model B1) and paternal male across all clutches (Model C1).

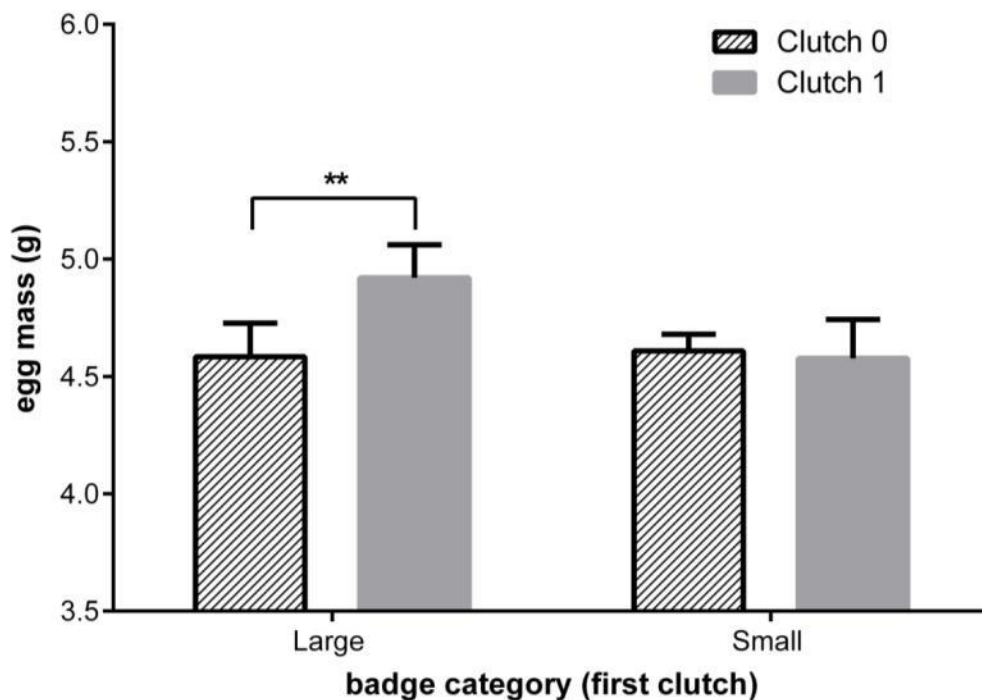
<b>Model A1- Clutch 1</b>		<b>Maximal Model</b>			<b>Minimal Model</b>		
<b>RESPONSE</b>	<b>EXPLANATORY</b>	<b>df</b>	<b>t</b>	<b>p</b>	<b>df</b>	<b>t</b>	<b>p</b>
Egg mass	Female baseline condition	2	3.18	0.086	3	0.07	0.948
	Clutch size	2	3.31	0.081	8	1.40	0.167
	Embryo sex	13	1.79	0.096	17	1.36	0.189
	<b>Male badge size</b>	13	-0.61	0.549	18	-2.38	<b>0.021</b>
	Male condition	13	2.53	0.024	16	1.73	0.102
	Male Mass	13	0.11	0.913	15	0.01	0.998
	<b>Interaction</b>						
	Female baseline condition*Clutch size	2	-3.30	0.081	2	-3.31	0.081
	Male badge size*Clutch size	13	1.06	0.304	14	1.13	0.274
	Male badge size*Embryo sex	13	-0.29	0.771	13	-0.29	0.771
<b>Model B1- Clutch 2, 3 &amp; 4</b>		<b>Maximal Model</b>			<b>Minimal Model</b>		
<b>RESPONSE</b>	<b>EXPLANATORY</b>	<b>df</b>	<b>t</b>	<b>p</b>	<b>df</b>	<b>t</b>	<b>p</b>
Egg mass	<b>Female baseline condition</b>	8	-0.48	0.639	16	-2.30	<b>0.050</b>
	Clutch size	8	-0.47	0.646	47	0.48	0.631
	Embryo sex	42	0.27	0.785	44	0.06	0.953
	<b>Initial Male badge size</b>	42	2.15	0.037	48	-3.49	<b>0.008</b>
	Initial Male condition	42	0.42	0.672	46	-0.11	0.918
	Initial Male mass	42	0.49	0.625	45	1.13	0.259
	Paternal Male badge size	-	-	-	14	0.08	0.939
	<b>Interaction</b>						
	Female baseline condition*Clutch size	8	0.49	0.631	8	0.52	0.612
	Initial Male badge size(Large)*Clutch size	42	-0.11	0.908	42	-0.11	0.908
	Initial Male badge size(Large)*Embryo sex	42	-0.46	0.641	43	-0.46	0.647
<b>Model C1- All Clutch</b>		<b>Maximal Model</b>			<b>Minimal Model</b>		
<b>RESPONSE</b>	<b>EXPLANATORY</b>	<b>df</b>	<b>t</b>	<b>p</b>	<b>df</b>	<b>t</b>	<b>p</b>
Egg mass	Female baseline condition	9	0.04	0.961	12	-1.20	0.253
	Clutch size	43	0.32	0.747	56	0.61	0.542
	Embryo sex	43	-0.29	0.771	50	-0.26	0.795
	Male badge size	9	-0.17	0.868	10	0.71	0.493
	Male condition	43	-0.28	0.777	45	-0.35	0.721
	Male mass	43	0.61	0.540	57	1.27	0.209
	<b>Interaction</b>						
	Female baseline condition*Clutch size	43	-0.31	0.761	44	-0.03	0.974
	Paternal Male badge size(Large)*Clutch size	9	0.43	0.671	9	0.46	0.655
	Paternal Male badge size(Large)*Embryo sex	43	-0.21	0.830	43	-0.21	0.830

**Table 4.3** Linear mixed effects models exploring clutch size in initial allocation (Model A2), the impact of initial male on subsequent clutches (Model B2) and paternal male across all clutches (Model C2).

Model A2- Clutch 1		Maximal Model			Minimal Model		
RESPONSE	EXPLANATORY	df	t	p	df	t	p
Clutch size	Female baseline condition	1	0.75	0.472	1	1.62	0.214
	Egg mass (mean)	1	2.03	0.081	1	1.84	0.316
	Male badge size	1	-0.56	0.600	1	-0.56	0.600
	Male condition	1	1.52	0.171	1	0.08	0.776
	Male mass	1	-2.16	0.066	1	-1.51	0.148
Model B2- Clutch 2, 3 & 4		Maximal Model			Minimal Model		
RESPONSE	EXPLANATORY	df	t	p	df	t	p
Clutch size	<b>Female baseline condition</b>	11	2.08	0.061	23	2.41	<b>0.024</b>
	<b>Egg mass (mean)</b>	11	2.13	0.056	23	2.49	<b>0.020</b>
	Initial Male badge size	15	-0.09	0.924	15	-1.48	0.158
	Initial Male condition	11	-0.14	0.884	12	-0.16	0.873
	Initial Male mass	11	0.41	0.689	13	0.47	0.643
	<b>Interaction</b>						
	<b>Female baseline condition* Egg mass</b>	11	-2.12	0.057	27	1.98	<b>0.025</b>
	Initial Male badge size*Egg mass	11	-0.07	0.940	11	-0.07	0.940
Model C2- All Clutch		Maximal Model			Minimal Model		
RESPONSE	EXPLANATORY	df	t	p	df	t	p
Clutch size	Female baseline condition	12	1.93	0.076	18	1.93	0.070
	Egg mass (mean)	12	2.03	0.064	19	1.93	0.069
	<b>Male badge size</b>	12	0.42	0.675	17	-2.15	<b>0.039</b>
	Male condition	12	1.39	0.187	14	1.45	0.167
	Male mass	12	-1.19	0.257	16	-1.69	0.109
	<b>Interaction</b>						
	Female baseline condition* Egg mass	12	-1.86	0.086	13	-1.81	0.092
	Paternal Male badge size*Egg mass	12	-0.69	0.499	12	-0.69	0.499

### 2.3.1 Initial allocation patterns

In the first pairing, females produced larger eggs if they were paired to a larger badge male (Table 4.2, Model A1). This was due to females increasing their egg size from their baseline egg size (egg mass prior to pairing) when paired to a large badge male;  $F_{1,57} = 11.01$ ,  $p = 0.002$ , whereas females paired with a small badge male for their first clutch showed no significant difference between baseline and first clutch egg mass;  $F_{1,38} = 0.03$ ,  $p = 0.870$ . Females paired with a large badge male for their first clutch ( $n=6$ ) had an average increase of  $5.55\% \pm 3.04_{SE}$  in egg mass from baseline eggs (pre-pairing) to pairing in first clutch whereas females paired to small badged males for their first clutch ( $n=6$ ) had an average decrease of  $2.29\% \pm 4.84_{SE}$  in egg mass from baseline eggs to pairing in first clutch (Figure 2.1).



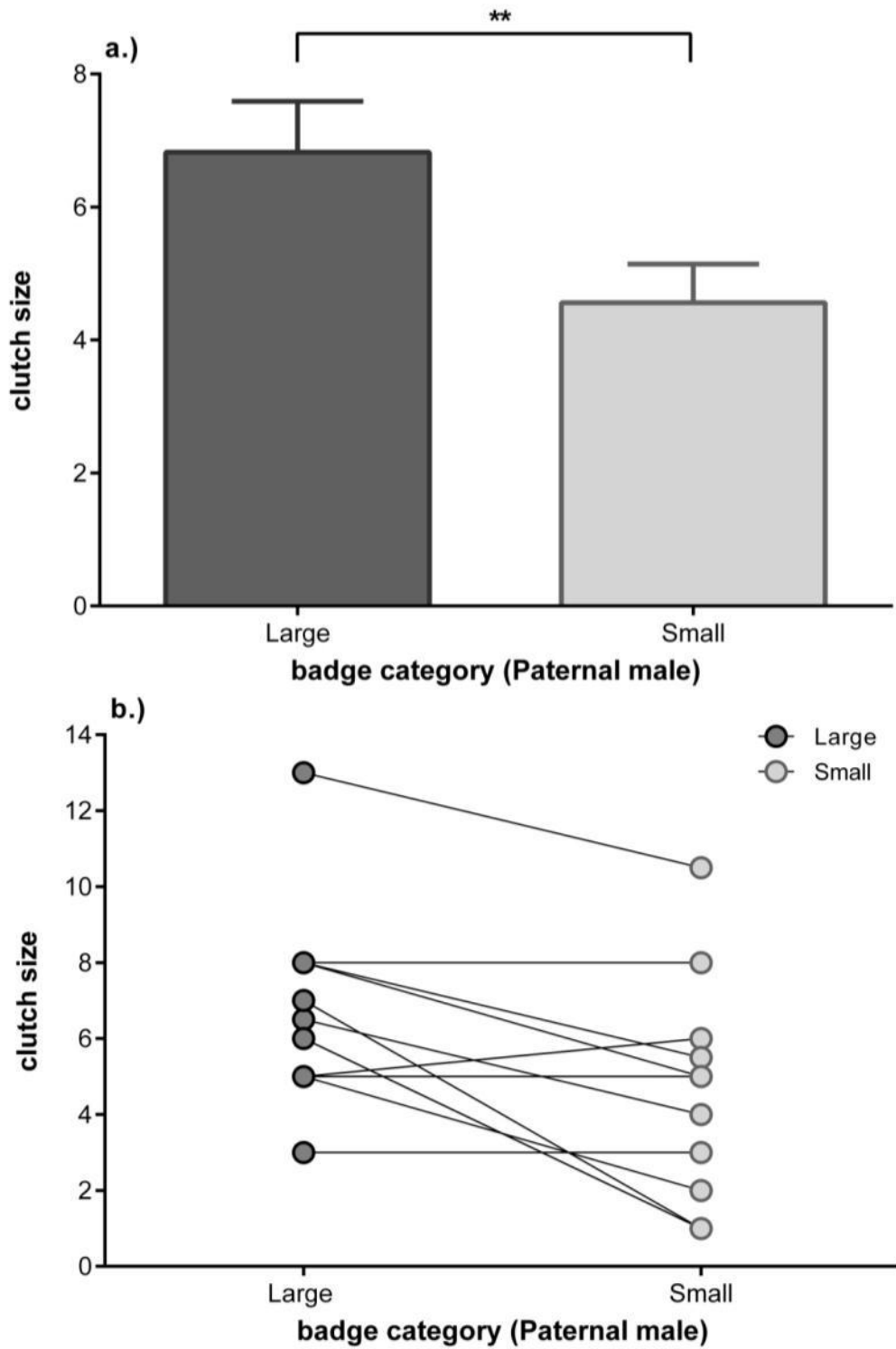
**Figure 2.1** Bar graph of egg mass means ( $\pm$  SE) - increase in egg mass (g) between pre-pairing (striped bars) with a male and post pairing with a male (solid grey bars).

The increase in egg size appeared to be due to an overall increase in investment when paired to large badged males as opposed to any trade off with clutch size as no interaction between clutch size and badge size was found (Table 4.2, Model A1). No significant interaction was found between embryo sex and male badge size in

relation to egg mass, showing females were not simply producing larger eggs as a result of producing more of one particular sex when paired to large badged males (Table 4.2, Model A1).

### **2.3.2 Effect of male traits on allocation across a series of clutches**

There was no effect of male traits on egg mass throughout a female's reproductive lifetime (Table 4.2, Model B1). However, there was evidence for differential allocation via changes in clutch size in response to male badge size;  $t_{17} = -2.15$ ,  $p = 0.039$  (Table 2.3, Model C2 and Figure 2.2a) with females producing larger clutches when paired with large badge males (Figure 2.2b). Male condition and mass had no significant relationship with clutch size. Across all four clutches there was no difference in the number of male and female embryos that developed;  $\text{Chi}^2 = 0.21$ ,  $\text{df} = 1$ ,  $p = 0.646$  and clutch sex ratio did not vary with paternal badge size;  $z = 1.08$ ,  $p = 0.277$ .

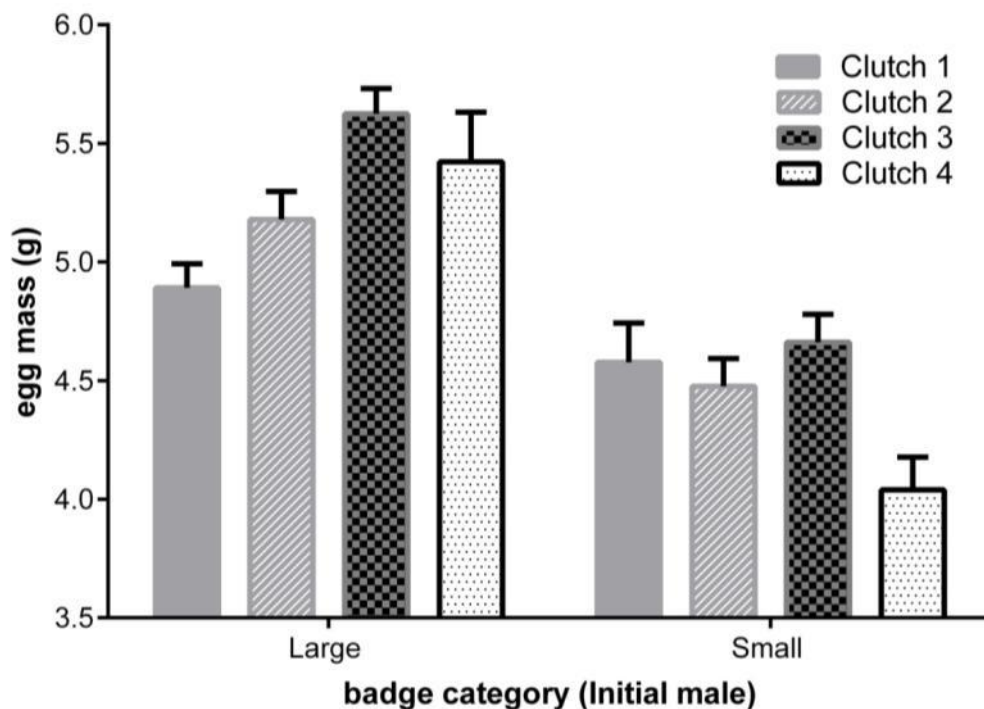


**Figure 2.2** Across all clutches females paired with a large badge male produced larger clutches compared to small badge males. Figure 2.2a mean clutch size ( $\pm$  SE) at group level, Figure 2.2b shows the change between pairings in clutch size at the individual level.



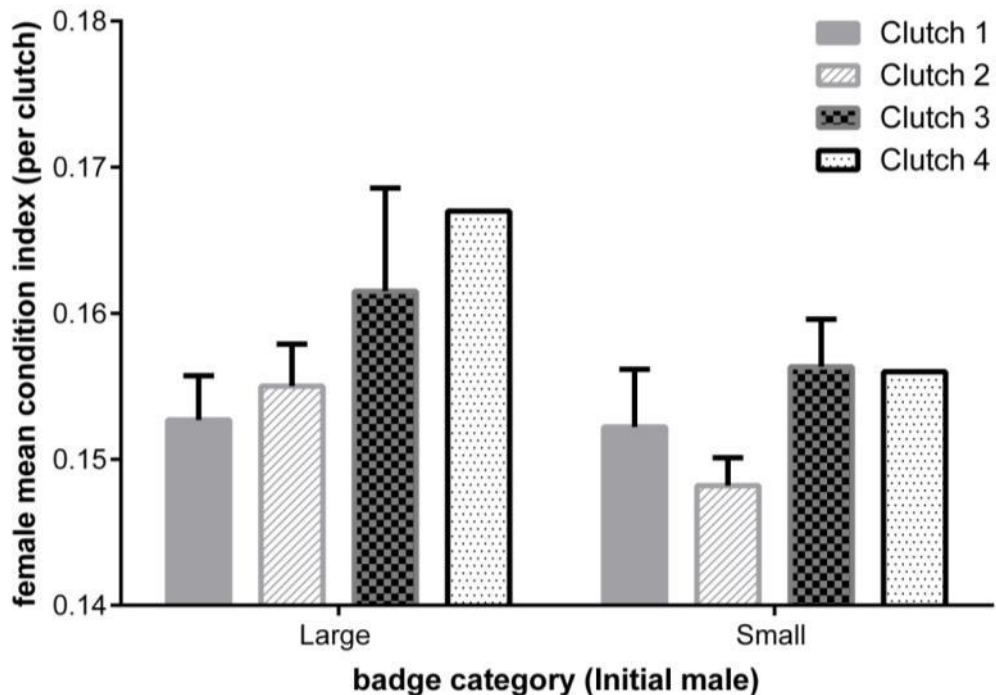
### 2.3.3 Carry-over effects on allocation of initial male badge size.

To establish if there was any carryover effect of initial allocation decisions across clutches 2, 3 and 4 I included initial male in the models. Interestingly initial male badge size was found to have a significant effect on egg mass across clutches (Table 2.2, Model B1). Female baseline condition also had an independent effect on egg size across all clutches (though there was no interaction between initial male badge size and female baseline condition (Section 2.3.5)). This highlights that initial male badge size is impacting on female allocation in subsequent clutches with other males and that females paired with a large badge male in their first clutch continue to produce large eggs across clutches 2, 3 and 4 (Figure 2.3). These findings were robust even when paternal male was included in the model (Table 2.2). Clutch sex ratio had no relationship with initial males badge size;  $z = 0.87$ ,  $p = 0.379$ . There was also no carry over effect of initial pairing on embryo sex;  $z = -0.25$ ,  $p = 0.802$  (or as an interaction with egg mass;  $z = -0.32$ ,  $p = 0.746$ ).



**Figure 2.3** Females initial paired to a large badge male continue to produce larger eggs (mean egg mass ( $\pm$  SE) compared to females initially paired with a small badge male;  $t_{48} = -3.49$ ,  $p = 0.008$ .

Initial male pairing also appears to influence female condition, which increases across clutches for females initially paired with large badge males;  $t_{11} = -2.21$ ,  $p = 0.044$  (Figure 2.4). No relationship was seen between Initial male badge size and female baseline condition;  $t_{11} = -0.50$ ,  $p = 0.624$ , therefore exploration of the change in egg mass from pre-pairing with a male (clutch 0) and post pairing was performed. Including both Initial male badge size and female mean condition across clutches in the model, change in egg mass was significantly affected by female mean condition over the experiment;  $t_{11} = 2.69$ ,  $p = 0.008$ , but was not related to first male badge;  $t_{10} = -1.24$ ,  $p = 0.244$ . This suggests that the carry-over effect seen on reproductive trait from first pairing may not be a direct effect of initial male badge and adjustment of allocation to it, but more of a secondary effect of the females ability to allocate driven by this change in female condition in relation to initial male badge size.



**Figure 2.4** Mean female condition ( $\pm$  SE) across females increase across clutches within females initial paired to a large badge male, compare to females initial paired to a small badge male in clutch one;  $t_{11} = -2.21$ ,  $p = 0.044$ .

Female mean condition index per clutch had no relationship with paternal male badge size;  $t_{21} = -0.06$ ,  $p = 0.948$ , male mean condition;  $t_{29} = 0.87$ ,  $p = 0.388$  or clutch order;  $t_{35} = 1.00$ ,  $p = 0.335$ . No residual effect of initial male badge size was found across clutches 2, 3 and 4 on clutch size however a non-significant positive

relationship was found with female baseline condition and clutch size;  $t_{27} = 1.98$ ,  $p = 0.056$ .

#### **2.3.4 Duration between reproductive attempts**

Duration between reproductive attempts did not differ significantly between clutches, (clutches one and two;  $3.7 \text{ day} \pm 0.482_{SE}$ , clutches two and three;  $6 \text{ days} \pm 1.19_{SE}$  and clutches three to four;  $7 \text{ days} \pm 1.81_{SE}$ ). There was also no significant effect of Initial male on the number of days between clutches;  $t_{10} = 0.51$ ,  $p = 0.619$ , or Paternal male;  $t_{10} = 0.45$ ,  $p = 0.658$ .

#### **2.3.5 Female traits**

Female baseline condition had a significant relationship with egg mass across clutches when Initial male was included in the model but did not have a significant affect across all models (Table 2.2). In clutches 2,3,4 when paternal male was not included in the model female initial condition has a significant relationship with clutch size. However when paternal male was included across all clutches there was no effect of any of the variables (female baseline condition, female mean condition across clutches) on the size of the first or subsequent clutches produced (Table 2.3). Nor was there any relationship between female mean condition and clutch sex ratio;  $z = 1.26$ ,  $p = 0.206$ .

### **Summary of Part A**

To summarise – I found that although females laid larger eggs when first paired to large badged males – they did not alter egg mass across clutches when paired to different males as would be predicted by DA theory. The lower baseline measurement demonstrates that females are both capable of increasing and decreasing investment in relation to male traits and that the lack of change over subsequent clutches is a true carry over effect rather than a complete lack of any mechanism for flexibility. Although females did not alter egg mass as predicted by DA – they did show differential allocation – but this was observed via changes in offspring number as opposed to changes in offspring quality. Finally, initial

allocation decisions influenced all subsequent breeding attempts. Females who were initially paired to large badge males obtained greater condition throughout the experiment and as a consequence continued to produce heavier eggs.

## **PART B**

### **2.3.6 Consequences of maternal allocation and paternal traits on embryonic viability and development**

To examine the effects of allocation decisions associated with male traits on offspring viability, all eggs were incubated artificially under standardised conditions to day 3. Eggs were then removed and the presence or absence of a viable embryo was recorded. The weight of the embryo at day 3 was taken and the embryo was sexed to identify if it was male or female, firstly, to account for sex differences in viability and secondly, examine the effects of paternal traits and female allocation on male and female offspring directly.

### 2.3.6.1 Egg viability

**Table 4.4** - Binomial mixed effects models exploring viability in initial allocation (Model A3), the impact of initial male on subsequent clutches (Model B3) and paternal male across all clutches (Model C3).

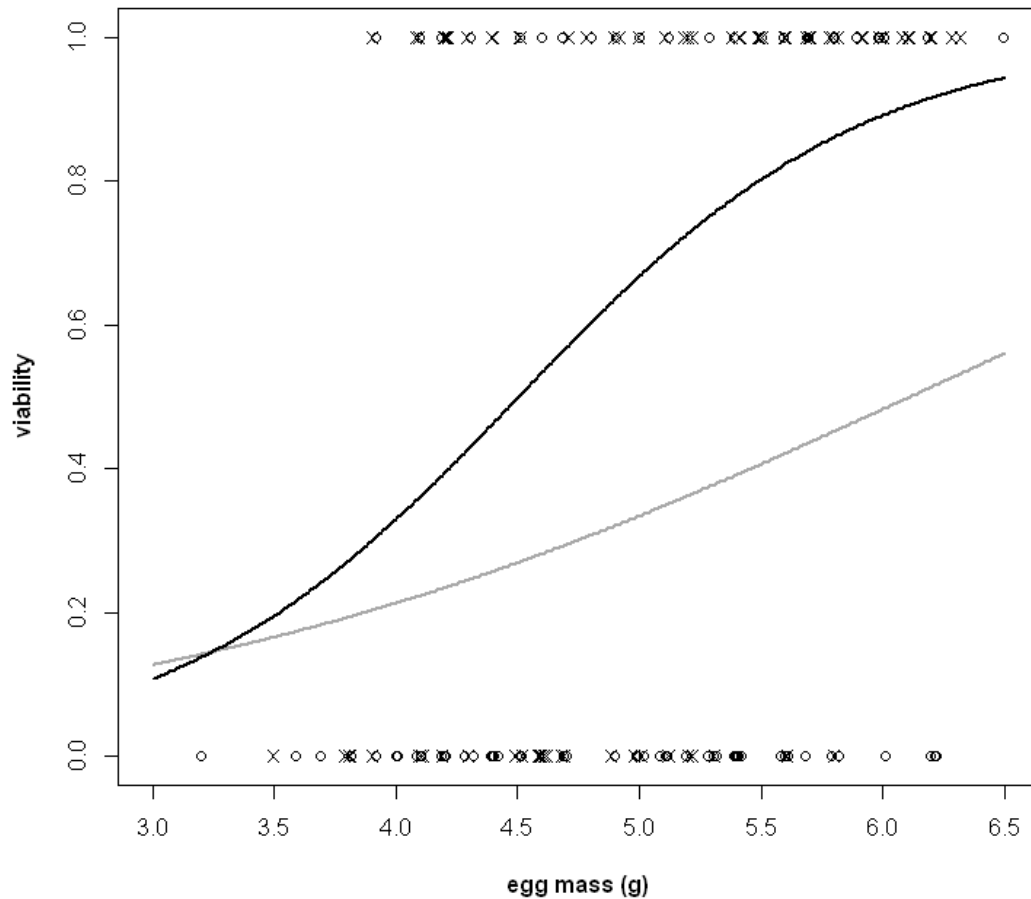
Model A3- Clutch 1		Maximal Model		Minimal Model	
RESPONSE	EXPLANATORY	z	p	z	p
Viability	Female baseline condition	0.85	0.391	-0.24	0.809
	<b>Clutch size</b>	-0.98	0.325	2.36	<b>0.018</b>
	Egg mass	0.69	0.487	1.49	0.135
	<b>Male badge size</b>	-0.35	0.724	-2.51	<b>0.012</b>
	Male condition	-1.24	0.213	-1.21	0.226
	Male mass	0.36	0.716	0.75	0.455
	<b>Interaction</b>				
	Male badge size*Egg mass	0.01	0.999	0.01	0.999
	Egg mass*Clutch size	1.11	0.267	1.46	0.144
	Female baseline condition*Egg mass	-0.89	0.369	-0.90	0.368
Model B3- Clutch 2, 3 & 4		Maximal Model		Minimal Model	
RESPONSE	EXPLANATORY	z	p	z	p
Viability	Female baseline condition	-0.04	0.967	-0.32	0.750
	Clutch size	-1.09	0.272	1.47	0.139
	Egg mass	-0.18	0.855	1.83	0.066
	Initial Male badge size	-1.59	0.109	0.88	0.377
	Initial Male condition	-2.01	0.091	0.07	0.978
	Initial Male mass	0.17	0.864	-0.02	0.984
	<b>Paternal Male badge size</b>	-	-	-3.07	<b>0.002</b>
	<b>Interaction</b>				
	Female baseline condition* Egg mass	1.26	0.207	-0.13	0.893
	Initial Male badge size*Egg mass	1.54	0.123	-0.49	0.667
Model C3- All Clutch		Maximal Model		Minimal Model	
RESPONSE	EXPLANATORY	z	p	z	p
Viability	Female baseline condition	-0.31	0.353	0.98	0.324
	<b>Clutch size</b>	0.22	0.701	2.07	<b>0.038</b>
	<b>Egg mass</b>	-0.31	0.387	2.52	<b>0.012</b>
	Male badge size	1.49	0.168	1.72	0.083
	Male condition	-0.84	0.323	0.74	0.395
	Male mass	-0.30	0.892	-0.43	0.663
	<b>Interaction</b>				
	Female baseline condition* Egg mass	0.50	0.613	0.52	0.596
	Egg mass*Clutch size	0.06	0.949	0.06	0.949
	<b>Paternal Male badge size*Egg mass</b>	-1.83	0.066	-2.30	<b>0.042</b>

#### **2.3.6.1.1 Viability of first clutch**

Male badge size was associated with egg viability in a female's first clutch (Table 4.4, Model A3) with females paired to large badge male producing more viable eggs than females paired with a small badge male. This appears to be a true association with male badge size as egg mass did not affect viability in this first clutch, either as an interaction between egg mass and badge size or between the difference in egg mass (from baseline egg size to first clutch egg size) and badge size (Table 4.4).

#### **2.3.6.1.2 Viability across clutches**

Across clutches, paternal male badge size has a significant effect on viability but only when paired to females laying larger eggs (interaction between male badge size and egg mass;  $z = -2.30$ ,  $p = 0.042$  (Table 4.4, Model C3 and Figure 2.5). Egg viability did not have any relationship with Initial male badge size as a main effect or as an interaction effect with egg mass, suggesting the carry-over effect of male badge size on female condition and female allocation does not directly affect viability in the first 3 days of development (Table 4.4, Model B3). There is a non-significant trend of change in egg mass interacting with paternal male badge size on viability;  $z = -1.72$ ,  $p = 0.084$ , this implies that females ability to increase her egg mass (potential reflecting her condition) may to some extent influence viability however, it appears this is not as big an effect as who a female is paired with at particular time.



**Figure 2.5** Viability probability between eggs from large badge paternal males (black line, crosses) and small badge paternal males (grey line, circles). Females paired to large badge males produce eggs more likely to be viable (1) than not (0) for a given egg mass (g), although this effect is smaller at smaller egg mass. Binomial logistic regression;  $z = -2.30$ ,  $p = 0.042$ .

### 2.3.7 Female effects on viability

Viability of eggs was not affected by female traits in the initial clutch a female produced (female baseline condition;  $z = -0.24$ ,  $p = 0.809$ , female mean condition;  $z = 0.402$ ,  $p = 0.687$  or baseline egg mass;  $z = 0.26$ ,  $p = 0.793$ ). However, clutch size had a positive relationship with the proportion of viable eggs a female produced;  $z = 2.36$ ,  $p = 0.018$  with females who produce a large clutch having a greater proportion of viable eggs compared to females who produce a small clutch. When females were paired to a large badge male they on average produced  $0.4\% \pm 0.155_{SE}$  more viable eggs compared to when they were paired to small badge males.

Therefore females on average produced  $0.5 \pm 0.95_{SE}$  more eggs when paired to a large badge males.

Across clutches there was no female effect on viability; female baseline condition;  $z=0.98$ ,  $p=0.324$ , female mean condition;  $z=0.97$ ,  $p=0.331$  baseline egg mass;  $z=0.24$ ,  $p=0.809$ .

### 2.3.8 Embryo mass

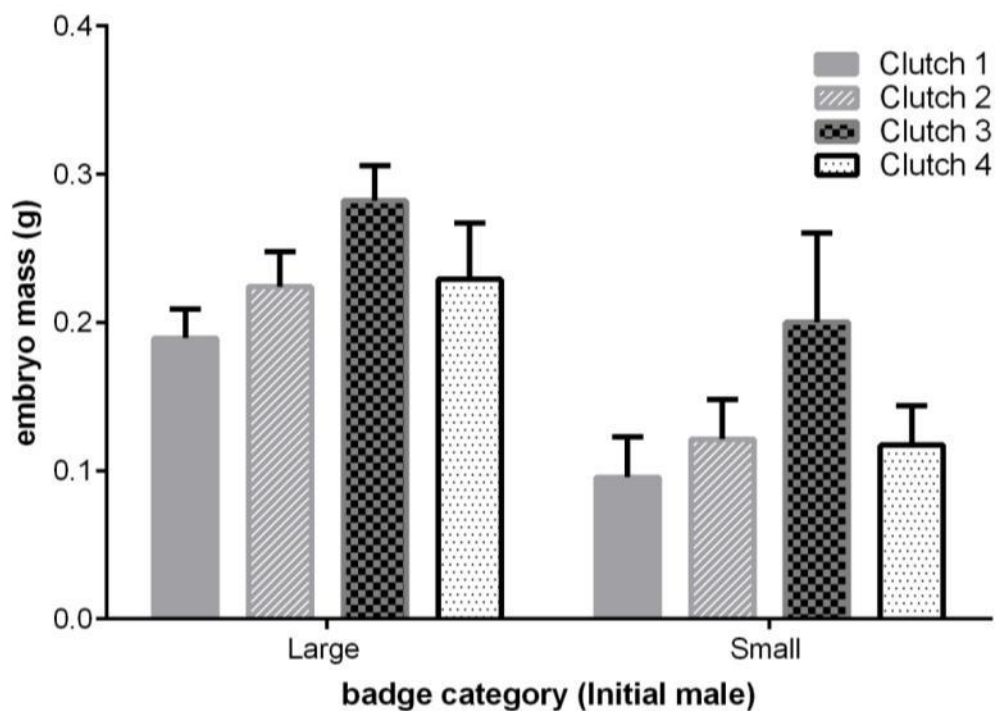
**Table 4.5** - Linear mixed effects models exploring egg mass in initial allocation (Model A4), the impact of initial male on subsequent clutches (Model B4) and paternal male across all clutches (Model C4).

Model A4- Clutch 1		Maximal Model			Minimal Model		
RESPONSE	EXPLANATORY	df	t	p	df	t	p
Embryo mass	Female baseline condition	2	-1.41	0.292	4	-1.69	0.165
	Clutch size	2	-1.35	0.308	3	0.16	0.882
	Embryo sex	13	-0.63	0.535	15	-0.31	0.763
	Male badge size	13	0.79	0.443	19	-1.72	0.101
	Male condition	13	0.05	0.961	18	0.47	0.646
	Male mass	13	-0.77	0.451	17	-0.77	0.449
	Interaction						
	Female baseline condition*Clutch size	2	1.35	0.307	2	1.18	0.359
	Male badge size*Clutch size	13	-1.06	0.308	14	-1.07	0.302
	Male badge size*Embryo sex	13	-0.04	0.962	13	-0.04	0.962
Model B4- Clutch 2, 3 & 4		Maximal Model			Minimal Model		
RESPONSE	EXPLANATORY	df	t	p	df	t	p
Embryo mass	Female baseline condition	47	-1.18	0.242	56	-1.69	0.095
	Clutch size	47	-1.09	0.281	55	-0.23	0.817
	Embryo sex	47	-0.85	0.398	50	-0.25	0.801
	<b>Initial Male badge size</b>	47	-0.01	0.990	6	-2.89	<b>0.027</b>
	Initial Male condition	47	0.81	0.419	53	0.49	0.620
	Initial Male mass	47	1.80	0.077	54	0.21	0.785
	Paternal Male badge size	-	-	-	52	0.61	0.545
	Interaction						
	Female baseline condition*Clutch size	47	1.08	0.281	48	1.11	0.271
	Initial Male badge size*Clutch size	47	-0.68	0.495	47	-0.68	0.495
	Initial Male badge size*Embryo sex	47	0.95	0.346	49	1.06	0.294
Model C4- All Clutch		Maximal Model			Minimal Model		
RESPONSE	EXPLANATORY	df	t	p	df	t	p
Embryo mass	Female baseline condition	51	-0.59	0.552	54	0.13	0.899
	Clutch size	51	-0.65	0.512	58	1.15	0.252
	Embryo sex	51	-1.41	0.163	55	-0.78	0.433
	Male badge size	51	0.29	0.768	59	0.77	0.444
	Male condition	51	-1.31	0.194	65	0.21	0.785
	Male mass	51	1.12	0.098	66	0.54	0.584
	Interaction						
	Female baseline condition*Clutch size	51	0.71	0.480	52	0.83	0.407
	Paternal Male badge size(Large)*Clutch size	51	-0.12	0.902	51	-0.12	0.902
	Paternal Male badge size(Large)*Embryo sex	51	0.18	0.240	53	1.07	0.287

The impact of male & female traits on reproductive allocation



Embryo mass in the first clutch was not affected by any of the factors or covariates investigated (Table 4.5, Model A4). This was also the case across clutches for paternal male badge size (Table 4.5, Model C4). However a carry-over effect was found of Initial male badge size where females originally paired with a large badge male are likely to produce heavier embryos;  $t_6 = -2.89$ ,  $p = 0.027$  (at 3 days of incubation), even when controlling for female mean condition for each clutch;  $t_6 = 0.21$ ,  $p = 0.834$  (Figure 2.6). Embryo mass had no relationship with embryo sex;  $z = -0.25$ ,  $p = 0.801$ .



**Figure 2.6** Females initial paired to a large badge male continue to produce larger embryos (mean embryo mass ( $\pm$  SE) ) compared to females initially paired with a small badge male;  $t_6 = -2.89$ ,  $p = 0.027$ .

### **Summary of Part B**

In summary, I found that male badge size was directly correlated with viability at the very earliest stages of development thereby identifying at least one benefit females may gain from mating with large badged males. Females benefit from altering their

allocation in clutch size when mated with these males as they produced proportionately more offspring as a result. Females may also obtain some form of direct benefit from mating with males with large badges as this alters their subsequent condition and allocation to individual eggs; this allocation has no effect on viability but does affect embryonic growth with females that had been mated initially to large badged males producing heavier embryos during early development as a consequence of early allocation changes. These effects carried over to subsequent clutches demonstrating the importance of considering previous experience when assessing the contribution of paternal genetic effects and female allocation effects on offspring development.

## **2.4 Discussion**

In this chapter I examine maternal reproductive allocation patterns across a number of reproductive events and their consequences on early viability and development. This study shows that female Chinese painted quail lay larger eggs for large badged males when they are first paired with a partner. However, allocation, at least in terms of egg size, does not then vary across subsequent reproductive attempts with different males, i.e. in this study no true differential allocation was found via change in egg mass. There is however true differential allocation in response to male traits via changes in clutch size with females laying more eggs for large badge males across a significant part of their reproductive lifetime. The effect of initial pairing also had consequences on later clutches with females that were first paired to large badged males consistently being in better condition and producing larger eggs than females that been paired to smaller badged males. However, not all females change their allocation to the same extent and their capacity to respond to these male cues appears to be mainly driven by female condition.

I then examined the consequences of these allocation patterns on embryo viability, growth and sex; viability appears to be closely linked to paternal male badge size and egg mass. Within this study I intended to explore female mortality and lifetime reproductive success however sample size limits interpretation of these results. It would be desirable to address these questions in the future to determine to what extent allocations impact on mothers over a lifetime. All allocation patterns were consistent with positive allocation when females were paired to a large badge “attractive” male and no evidence for any disadvantage of being paired to a small badge male or any associated compensation effect was found. To my knowledge this is the first study to explore differential allocation in reproductive traits across more than two clutches and its consequences for early embryonic development.

### **2.4.1 Female allocation decisions**

Initial pairing with a male (in clutch 1) had a significant relationship with egg mass, where females alter their allocation by increasing or decreasing it from their baseline

level, dependent on the male they are initially paired up with. This pattern has been reported in other studies of this species and assumed to be indicative of differential allocation (Uller et al., 2005). However, it was found instead that females do not alter egg size when paired to subsequent males but instead alter the number of eggs they produce in response to male characteristics. This suggests that allocation mechanisms used by females may vary across different stages of a female's reproductive lifespan. However, egg size did play an important role across clutches in another way - when females were re-paired they continued to produce eggs of a greater size if originally paired with a large badge male or lesser size if originally paired with a small badge male and a similar relationship was also found with embryo mass. This carry-over effect may be due to traits associated with an initial partner signalling benefits such as resource availability, or the pairing with a high ranking male early in reproduction may alter females' social status which may then impact on subsequent allocation. Effects of a first male's sperm are unlikely to be a mechanism influencing such carry over effects as it is likely that little to no sperm from the previous male would be present in subsequent clutches; mating diminishes quickly after pairing (personal observation) and average sperm storage times are generally shorter than the time taken to complete the laying of the next clutch (Cunningham unpublished data). However, male manipulation of female investment should not be completely discounted as the effect of male ejaculate components on female behaviour and physiology in birds has not been fully explored. Importantly, in this study I highlight that female condition over the experiment had a significant relationship with initial male badge size. When the mean female condition was included (not baseline condition which accounts for female condition pre-pairing) in the analysis this explains more about the change in egg mass than initial male badge size. This suggests that initial pairing has a physiological impact on females and that effects seen after this pairing may not be an allocation decision but instead a residual effect of this physiological change (Rutstein et al., 2005). To my knowledge this is the first study to show that this type of effect persists across several breeding attempts, highlighting the potential importance of considering early experience and initial mate choice decisions and their impact on allocations and fitness measures later in life.

### **2.4.2 Differential allocation**

Although egg mass did not change across breeding attempts, clutch size did – with females producing more eggs when with large badged males providing evidence for differential allocation. The result meets the basic assumptions of differential allocation theory, that females are allocating differentially and this is seen across the four male pairings tested, illustrating the plastic nature of their response to male cues. The result is also robust as females were paired to both attractive and unattractive males allowing us to control for female effects on allocation. Other studies have found similar results. For example, in a study the total mass of train in peacocks (Petrie and Williams, 1993) had a significant positive relationship with total number of eggs. In zebra finches females paired to preferred mates (of higher song duration and frequency) produced a greater clutch size (0.5 eggs more) with preferred males, while other reproductive traits appeared relatively non-plastic (Balzer and Williams, 1998).

This study demonstrates the importance of exploring male effects on reproduction by exploring multiple breeding attempts. In another study using Chinese painted quail (Uller *et al.*, 2005) the authors found no effect of male badge size on clutch size but did find that egg mass was affected by male badge size. However, this was only across one clutch and therefore it is unclear what the authors would have concluded if the experiment continued onto more reproductive events. It has been hypothesized that diversity seen among species in differential allocation to reproduction, e.g. between findings such as egg mass, clutch size or egg component alteration, comes from adaptive differences in systems of parental care (Horvathova *et al.*, 2011). In a recent review (Horvathova *et al.*, 2011) the authors suggests that differential allocation between species with minimal male parental care may result in females allocating differentially to her clutch via egg mass. However, in species where there is biparental care, differential allocation may be through clutch size alteration. Systems of parental care may be of significance when exploring what reproductive traits are being influenced by differential allocation, it may also be important to consider (based on the findings in this chapter) what reproductive event an individual

is in to whether predictions will be for differential allocation on particular reproductive traits. Experience can play an important role in reproductive success and in wandering albatross (*Diomedea exulans*) there is a particularly large improvement after a female's first breeding attempt (Froy et al., 2013). With success increasing with experience one could hypothesis that the number of offspring produce rather than the amount of prenatal provisioning per clutch member may have a greater effect on an individual's reproductive success through time.

### **2.4.3 Consequences of allocation and paternal traits**

In this study an effect of paternal male badge size was found on viability. This could arise because male badge size is a secondary sexual characteristic reflecting male fitness (or quality of resources available to him) and be one basis for why it may pay females to invest more in reproduction (in terms of increasing egg number) when paired to these males. However, in this study there was no experimental manipulation of male badge size, therefore the relationship found between embryo development and male badge size could be due to female allocation of other important constituent within the egg that are not detectable through changes in egg size, or male quality. A study using dung beetles goes in some way to pull apart these two traits of maternal allocation and male genetic contribution to determine the cause of differences in viability (Watson and Simmons, 2012). The study first finds evidence for differential allocation to particular males (males with high courtship rate) via increased size of the brood ball produced. The study then goes on to experimentally manipulate female maternal allocation by changing the size of the brood ball. The results indicate that differential allocation only has a small effect of viability (number of eggs surviving to adulthood) and the main contribution to viability appears to be due to male quality. The study determines that male genetic quality results in increased/decreased offspring survival to adulthood and that maternal allocation impacts on offspring characteristics related to its success e.g. offspring body size and horn size. If embryo viability is a consequence of male effects that are signalled by male badge size in Chinese painted quail, females would benefit from investing more in these males. The effects of male traits and maternal allocation on embryo traits as opposed to post-hatching traits have been far less

explored. The findings suggest they play a significant role in overall measures of parental success via the number of eggs being produced and the viability of these eggs.

#### **2.4.4 Female investment and causes for variation**

It is well documented that maternal condition is linked to reproductive allocation (Parker, 2002, Smith et al., 1993, Nager et al., 1999), and the results from this study are in accordance with this. Females of higher condition produced eggs of a larger mass than females of lower condition.

Considering female condition components including baseline condition and condition post pairing has, in this study, allowed us to explore not only the effect female general condition has on allocation decisions but also to determine that maternal condition can be affected by external cues such as male badge size. This in turn allowed us to identify that the carry-over effect on egg mass of the initial male pairing is likely to be a secondary effect and that initial male badge size influences female condition across the experiment. This may then be the mechanism by which initial male effects egg mass. Within the literature there is evidence for females altering their allocation due to male traits via their own condition, where females increase allocation in response to male attractiveness only if she is in good condition (Rutstein et al., 2004). The literature has suggested that initial reproductive encounters can impact an individual's condition throughout its life and this could be what is occurring here. Age (Brommer et al., 1998) and condition –Blue Petrel (Chastel et al., 1995) at first reproduction can have an effect on females life time reproductive success and with a semi-monogamous species such as in this study, longer lasting effects of initial pairing could also have a similar knock on effect. Female short term preference has been found to be linked to the “attractiveness” of a recently experienced male (Collins, 1995), with females adjusting their preferences based on this exposure. Therefore it's not uncommon for early pairings to determine something about later reproduction but this study provides the first evidence that allocation decisions can be shaped in the same way.

Furthermore, the carry-over effect of early allocation decision had an impact on embryo weight at day 3; these effects may persist through to hatching as has been observed in other species (Cunningham and Russell 2000) and hatching condition can play a key role in early survival across species (Bolton, 1991, Gorman and Nager, 2004, Pearce-Higgins and Yalden, 2002). Female experience and current reproductive investment may be linked depending on the amount of time between reproductive attempts and or how flexible females are in allocation should be taken into account when exploring differential allocation in relation to multiple breeding attempts.

#### **2.4.5 Conclusions**

This study shows that Chinese painted quail vary their allocation depending on their partner and that sexual ornamentation (badge size) is an important trait for female allocation decisions. However due to the cross-over design implemented, the finding here gives a clear picture of the importance of partners on allocation and that timing of pairing can play an important role. Carry-over effects of early allocation due to initial male badge size was found on egg mass, and this persistence appears to be due to change in maternal condition (influenced by this initial male pairing). These findings illustrate the importance of early reproductive experience on reproductive traits as well as partner traits.



# 3

## CHAPTER

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### **The impact of parental traits on the transfer of maternal antibodies**

#### **3.1 Introduction**

##### **3.1.1 Maternal effects**

Maternal effects that influence reproductive allocation of resources can have a major effect on offspring development and can potentially be influenced by both environmental and social conditions (see Chapter 2). Females may allocate resources to a particular reproductive attempt by altering traits such as egg size and egg number based on its likelihood of success. However, these female allocation decisions may also alter the individual egg components that play a crucial role in the embryos early development such as hormonal, nutritional and immunological components. For example, when egg size is adjusted in response to male traits via differential allocation, it is not clear whether all egg components are altered proportionately. Females may also potentially alter the level of different egg components completely independently of egg size. These possibilities are important to establish as variation in these components can potentially have both positive and negative effects on offspring success.

##### **3.1.2 Maternal antibodies**

One of the key components that may be affected by maternal allocation decisions are maternal antibodies. Maternally transferred antibodies are believed to be vital for many species due to an offspring's vulnerability to infection in the early stages of life: In birds (as well as mammals) the immune system develops after birth, therefore there is a period where offspring are at high risk from pathogens. Young birds focus

a considerable amount of energy into growth at this stage and therefore the additional cost of mounting an immune response may be highly detrimental at this point. Vertical transmission of antibodies from mother to offspring can therefore potentially reduce the cost of immune defence (Rose and Long, 1971, Staszewski et al., 2007b). Equipped with maternal antibodies, offspring are prepared with specific antibodies for the pathogen environment they are being born into. This transfer of specific antibodies can play an important role in the survival of populations through epidemics, and survival of such early infection can lead to long lasting protection (Navarini et al., 2010). The amount transferred to offspring can also have a significant effect as the more antibodies that are transferred the longer the protection is to the offspring (Grindstaff, 2010). However, this has to be balanced with any potential negative effects associated with high antibody levels in the offspring. In some cases where maternal antibodies are present, an effect known as “blocking” can occur. The blocking effect is where an individual’s immune response is effectively blocked from responding due to maternal antibodies interacting with the antigen involved. This can sometimes be viewed as beneficial as the offspring does not mount its own immune response, therefore allowing resources to be used for other processes (Staszewski et al., 2007a, Grindstaff, 2008). However, if the humoral immune response is blocked it is not prepared the next time the offspring is exposed to the pathogen (when maternal antibodies are not present); it will then have to use the primary immune response, which is more costly, slower and not as strong as the secondary response. The optimal level of transfer to maximise offspring fitness is therefore likely to reflect a balance between these effects.

### **3.1.3 Differential allocation of maternal antibodies**

Differential allocation is the allocation of resources to different offspring or reproductive attempts according to its likely success in order to maximise an individual’s own reproductive fitness (Sheldon, 2000). Maternal transfer of immunity may be influenced by these allocation decisions, either directly, because it may pay females to vary immunological allocation, or indirectly, as a consequence of other allocation decisions. However, testing this empirically is complex given the complexity of the immune response. Unlike other maternal resources adult birds do

not consistently have a supply of antibodies, nor can they trigger specific antibody production without having had recent contact with a pathogen. Therefore the relationship mothers and offspring have with antibodies will be environment dependent. Furthermore, if a mother is responding to a pathogen and producing an antibody response, levels seen in offspring produced over this time may simply reflect this changing immune response. These sources of variation need to be accounted for in studies examining the benefits and ability of a female to transfer these antibodies to offspring, as they may vary considerably between environments and populations.

### ***3.1.3.1 Differential allocation of maternal antibodies due to male traits***

In avian systems there appears to be no vertical transmission of immunity from father to offspring and paternal antibody levels have been found not to correlate with egg or chick antibody levels (Gasparini et al., 2002). Hence, any change in transfer found in relation to a mate characteristic is generally assumed to arise as a result of differential allocation by the female. Paternal attractiveness has been suggested to impact on a female's allocation of resources, however only a small number of studies have explored allocation of maternal antibodies in relation to paternal traits (Saino et al., 2002b, Hargitai et al., 2006). In the first study, wild female barn swallows paired to perceivably more attractive males (some of whom were artificially increased or decreased in attractiveness) transferred more antibodies to eggs (Saino et al., 2002b), however others have found no effect of male traits on transfer, for example, female Collared Flycatchers were found to not alter yolk antibody concentration in relation to male traits (though total antibody content is unknown) (Hargitai et al., 2006).

There are a number of ways differential allocation of antibodies could occur; firstly, males may induce a change in female antibody levels and differences found in eggs may be as a consequence of passive transfer of higher or lower level of antibodies from the female. Secondly, female antibody response could remain unaffected but the relative amount transferred is altered or thirdly, changes in antibody level or

concentration could be a by-product of changes in allocation in general, for example, a bigger egg could have proportionately more antibodies or same level of antibodies but at a lower concentration and only knowing yolk size would allow for these possibilities to be untangled.

Some studies have found sex differences in egg components however, it is unclear if these differences occur because of different allocation levels or because the sexes utilise different levels of egg constituent. There are two studies that have explored maternal antibody transfer in relation to offspring sex (Saino et al., 2003, Martyka et al., 2011). In the first, authors found in zebra finches that sexes varied in the level of antibodies present in relation to laying order (Martyka et al., 2011) and the interaction between sex and laying order came from a significant decline in maternal antibodies over a clutch in male offspring (no significant change over a clutch in female embryos). Females may allocate more antibodies to male offspring, firstly because they may be the more valuable of the sexes to female reproductive fitness following a mating with a preferred male i.e. this could be related to differential allocation. However, male offspring may have greater levels of testosterone or other immune suppressing substances and therefore females may allocate more to them to compensate for this. In the second study using barn swallows the authors found female embryos had higher concentrations of antibodies compared to sons (Saino et al., 2003). However, both studies sample yolks after embryos had begun development, therefore it is unclear if differences are arising pre or post incubation- there may be differential catabolisation by the developing sexes so that antibodies are lost from the egg environment during early incubation. This is highly important to determine as if it is the latter this would show that there isn't differential investment at work but instead a subsequent physiological process causing variation.

### ***3.1.3.2 Egg development and maternal antibodies***

While variation may be occurring due to allocation decisions and trade-offs, it is important to determine how antibodies become present in eggs and yolks. The

transfer of antibodies from mother to offspring prior to birth occurs in two parts. The first step in this transfer is from the mother's circulation into the developing yolk.

Yolk development in quail and chicken is sequential unless females prepare to incubate. At daily intervals single oocytes enter a two-phase maturation process, where there is active uptake of proteins from the maternal blood stream (Schneider et al., 1998). The first phase has slow growth and lasts ~15 days (~ 21 to 6 days before lay). The second has rapid growth where the yolk increases in size 30-50 fold in mass and the process lasts ~6 days (~ 6 to 0 days before lay). Once the oocyte is ovulated it moves into the infundibulum where fertilisation occurs. The fertilised oocyte then begins its passage down the oviduct where other components of the egg such as albumen and shell are added over the next 24 hours. The fully formed egg is laid 24 hours after fertilisation and the next ova with a fully formed yolk is then released into the infundibulum to be fertilised and will be laid fully formed 24 hours later.

The transfer of antibodies to developing yolks follows a similar pattern to yolk size increase. Firstly low levels of antibodies are transferred to the yolk as it develops in the ovary. At oogenesis, there is then a sudden influx of antibodies and other yolk components in the last few days prior to egg formation in the reproductive tract and laying (Kowalczyk et al., 1985). The transfer to the actual embryo from the yolk then mostly occurs in the few days prior to hatching by receptor-mediated transfer across the yolk membrane into the embryos blood (Kowalczyk et al., 1985). For maternal antibody transfer from mother to yolk the first part of this mechanism is believed to be receptor mediated and individuals may differ in the number and effectiveness of this transfer process. However, from the literature the transfer of antibodies also appear to be directly correlated to the increase in size of yolk as an egg is formed (Kowalczyk et al., 1985) so investment may be tied to allocation of other constituents laid down in the last few days of egg formation. The exact timing of these events is largely unknown, information to date is from chickens but it is unknown if the timings are similar in other species.

It is well documented that females increase their antibody production when eggs are being produced (Klasing, 1998), therefore maternal investment is likely to incur some cost because of the increase energy demand the individual is under. Above that, it is unknown if the mechanism required for transfer is also an expensive process and if so these extra drains on resources could become highly influential at this expensive phase of life. Therefore, resource limitation impacting on female condition has been stated as a mediator of variation in maternal antibody transfer (Grindstaff et al., 2003). While many studies have explored the effect of maternal condition on the level of antibody transfer few have examined the cost of this transfer and it's affect on other female traits related to her fitness. The lack of evidence in the literature of a direct cost of transfer may be that female's condition mediates antibody transfer and therefore no further trade-off occurs. Nutritional state could also lead to variation in production and transfer of antibodies; however this may depend on the type of antibodies being explored. For example, Blount found total plasma antibodies was significantly decreased in individuals fed carotenoids, resulting in these individuals transferring lower levels of antibodies to their eggs (Blount et al., 2002). Whilst it is believed there is a cost of producing one's own immune response in relation to other traits, there is little information as to if there is an additional cost of transferring antibodies for mothers and whether this cost will vary in response to the level they allocate.

The pattern of antibody production and transfer of these antibodies to eggs for a specific pathogen is likely to be related to the timing of challenge or infection. However other "adaptive" responses (differential allocation) may also be occurring over a clutch, therefore controlling for variation in maternal levels is vital for untangling the effect of time and any other adaptive traits on allocation over a clutch. Here I test firstly at what point antibodies are transferred to the egg to establish how far in advance to laying any social interactions would have to operate to induce any change in female allocation. I then examine whether females 1) vary in their own antibody response in response to male traits, 2) vary the transfer level or alter their allocation of immunity in response to male traits, 3) whether differences in allocation in egg mass result in a proportional increase or change in concentration of antibody

level 4) vary their allocation over a clutch, 5) vary in response to sex and 6) whether all females follow a single pattern. Finally 7) I look at the cost of this production and transfer in the absence of a pathogen challenge. I develop a novel non-destructive approach for testing antibody levels in eggs at different points of development to monitor if the catabolisation of levels vary across development for the different sexes or different members of a clutch.

## **3.2 Methods**

The study was conducted using a colony of Chinese painted quail (*Coturnix chinensis*), housed at the University of Edinburgh. General husbandry procedures and the production of maternal generation birds were as outlined in section 2.2.1.

### **3.2.1 Male morphological traits**

Male traits were measured in order to rank males on the basis of trait size before assigning them to females. Chinese painted males have a distinct black and white patch on their throats (referred to as a “badge”) and females use the size of this badge upon which to base allocation decisions, as found in Chapter 2, and see (Uller et al., 2005). Measurements of overall badge size and the relative areas of black and white components of the badge were achieved by taking standardized photographs of each bird as described in chapter 2. Measurements were repeatable over time (repeatability; front white ( $r=0.94$ ), front black ( $r=0.77$ ), side white ( $r=0.82$ ) and side black ( $r=0.70$ )). No males moved between large and small classification between badge size measurements.

The mass of all males were measured to the nearest 0.1 grams every four days during the experimental period. Pectoral muscle and fat were assessed using BTO guidelines (Ringers’ Manual BTO, Thetford). A skeletal morphometric was determined using the tarsus length with an accuracy of 0.01 mm using electronic callipers. As this was an experimental set up with birds kept under standardized conditions, body condition index was calculated by  $\text{mass} / \text{tarsus length}^3$ , though different measures of condition revealed qualitatively similar results (see, Galvan, 2010, for discussion).

### **3.2.2 Female morphological traits**

Female morphometrics including tarsus length, weight, muscle and fat scores were measured as detailed above. A female’s baseline egg size was established prior to the experiment by taking the weight, length and width of all eggs laid prior to housing with any given male and calculating the average weight and volume of an egg laid prior to pairing.



### **3.2.3 Experimental setup and treatment groups**

#### **3.2.3.1 Housing**

Birds were housed throughout the experiment in their experimental pairings in breeding cages (800 x 500 x 375 mm). Each breeding cage was lined with wood shavings, and contained a nest area for the female (covered area containing hay) and a communal sand bath. Cages also contained artificial foliage and one food and water hopper.

#### **3.2.3.2 Female treatment**

Biometrics of birds (total body mass (g), tarsus length (mm)) were measured and baseline blood samples were collected on day -1 of the experiment. On day 0 of the experiment prior, to pairing to a male each female, was randomly assigned to one of two treatment groups: a negative control group was injected with 0.1ml of phosphate-buffered saline (PBS) (n=15), the experimental group was challenged with 0.1ml inactivated *Salmonella* vaccine (SalenvacT, Intervet) (n=15). A control group was included in the experiment to compare the effect of vaccination on female allocation, condition and egg production. There was a significant difference in the level of antibodies present between the two groups;  $F_{1,20} = 9.06$ ,  $p = 0.006$  and control females did not produce Ab levels above that of the positive threshold. All models exploring mother or maternal antibodies only include treated *Salmonella* vaccinated individuals.

#### **3.2.3.3 Male pairing**

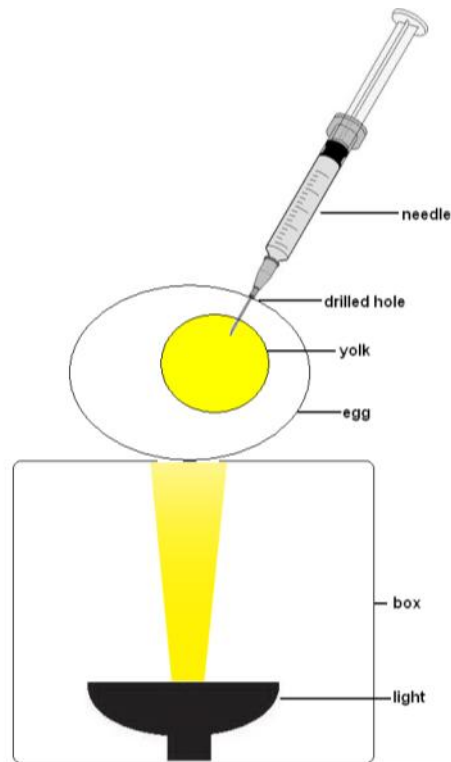
Eight of the PBS and SalenvacT female treated females were assigned to be paired initially with a large badge male in their first clutch and seven females from each treatment group were assigned to be paired initially to a small badge male. Pairs were left undisturbed to initiate and lay a full clutch of eggs. Once egg laying started, eggs were removed on a daily basis but replaced with an identical dummy egg in the same location as the egg was found to induce females into laying a natural clutch. At day 15 of the experiment, males were removed from their partners and the dummy clutch of eggs removed to induce females to lay a further clutch of eggs. Males were

then allocated to another female's cage; females previously housed with large badge males were housed with small badge males and vice versa; This was repeated a further two times to collect four clutches of eggs from each female – two from pairings with large badged males and two from pairings with small badged males.

### **3.2.4 Female sampling, egg measurements and antibody sampling pre-incubation**

Female sampling: Blood samples were taken immediately prior to challenge then every 4 days up until 60 days post-vaccination. Samples were collected via puncture of the metatarsal vein using a sterile needle and collection of the blood using 100µl capillary tube. Blood samples were centrifuged on day of collection at 9050 rcf/g for 10 minutes and serum collected and stored at -20°C until antibody analyses were conducted. All work was conducted under Home Office License (No PPL 60-4115), with full ethical approval from the University's ethical committee and with veterinary supervision throughout.

Egg sampling: Eggs were collected on a daily basis and mass, length and breadth were recorded at time of collection. Chinese painted quail eggs can be candled by placing a bright light source behind the egg to show the position of the yolk through the shell. Eggs were placed in the same position on a light box to illuminate the yolk; the yolk sac has a large volume and is usually centrally positioned. Using a micro electric drill each egg was drilled (at approximately the same position) on the side of the egg to produce a small hole (Image 3.1). A 27G needle attached to a 0.5 mL syringe was inserted in to the hole and into the yolk. Yolk (5µl) was extracted via the hole using a syringe and needle and then frozen at -20°C for antibody analysis. The egg was then sealed using melted wax and incubated.



**Image 3.1- Pre-incubation yolk extraction method.**

### **3.2.5 Embryonic development**

After antibodies had been extracted and the egg sealed, eggs were directly transferred into incubators with automatic turners; to be incubated under standard conditions (37-38 °C and 40-50% humidity). After 3 days, eggs were removed from the incubator and stored at minus 20°C until further analysis was conducted. Eggs were then carefully dissected and the presence or absence of embryos was recorded. All egg components were then weighed including the embryo mass (if present), yolk mass and albumen mass. If an embryo was present this was put aside for sexing and yolks were kept for further antibody analysis.

DNA was extracted from embryo samples using *DNeasy kit (Qiagen)*. The samples were then sexed using the polymerase chain reaction (PCR) to amplify part of the W-linked avian CHD gene (CHD-W ) in females, and its non-W-linked homologue (CHD-Z) in both sexes, using PCR primers 2718R and 2550F (Fridolfsson & Ellegren 1999). Products were run on a 2% agarose gel and visualized with ethidium bromide and the presence of either one (CHD-Z; males) or two distinct bands (CHD-

Z and CHD-W; females) were used to determine sex. All eggs that developed a visible embryo were successfully sexed (SalenvacT; n=31, PBS; n= 69).

### **3.2.6 Dissection and developing oocyte sampling**

At the end of the experiment the 6 females who were still alive at the end of the four clutches were weighed and blood sampled to determine plasma antibody titre then killed (using Schedule One methods). Dissections were then performed on each female to collect all developing oocytes and follicle clusters. All distinguishable oocytes were weighed and yolks extracted, undefined oocytes were weighed as a cluster and yolk antibodies extracted. Extraction of yolk was performed by cutting the oocyte in half and squeezing out the yolk. Of the 6 females dissected 4 still had above positive levels of antibodies in their plasma, however one female did not (female 466). Another female also does not fit with the oocyte mass increase (female 529), suggesting she may have begun to stop laying. Both these females were removed from analysis. Egg follicles develop in the order in which they will be laid and each egg is laid at 24 hour intervals. The largest follicle present is therefore in the day prior to release into the top of the reproductive tract where fertilisation occurs. The egg then has albumen and shell added over the next 24 hours to be laid a day later. The largest follicle present is therefore measured 2 days prior to lay and is called -2, the next largest is 3 days prior to lay and therefore called -3 etc. After day -4 oocytes samples were pooled so that enough of a sample for antibody extraction could be collected; oocytes smaller than the -4 days oocytes to be laid were pooled into; pool1, pool2 (slightly smaller, less defined than pool1) and cluster oocytes (lack of colour, hard to define individual oocytes).

### **3.2.7 Antibody extraction**

Antibodies from yolk extracted pre incubation and post incubation were extracted following Mohammed protocol (Mohammed et al., 1986). The homogenized yolk was diluted 2:1 in Phosphate- buffered saline (PBS) and vortexed for 2 minutes. Chloroform was then added to the egg yolk/ PBS mixture at a 1:1 ratio, and vortexed for a further 2 minutes. The mixture was then centrifuged on 9050 rcf/g for 10

minutes. After this time the mixture separates into three layers; a top layer containing PBS and supernatant (used for antibody analysis), a deposit of fatty lipids in the middle layer and an organic phase containing chloroform and carotenoids in the bottom layer.

### 3.2.8 Antibody analysis: Enzyme-linked immunosorbent assay (ELISA)

Specific enzyme-linked immunosorbent assay (ELISAs) was performed for treatment and control groups. A *Salmonella*-specific ELISA test was performed using FLOCKTYPE® *Salmonella* ELISA kit (Labor Diagnostik Leipzig, Germany). The kits were manufactured for chicken serum and plasma but quail antibodies are also detected and a number of studies have used anti chicken antibody to detect antibodies in various quail species. Previous pilot work had established the appropriate dilutions to ensure antibodies lay within the bounds of the detectable range of the ELISA kits and dilution curves confirmed these lay within the linear part of the test.

Yolk extractions were diluted 1:62 and blood samples were diluted 1:124 with the buffer provided. The optical density (OD) of the resulting solution was read in a spectrophotometer at 450nm immediately after stopping the reaction, and corrected for using positive and negative controls supplied in the kit. To estimate the repeatability of the method, one sample for each of the treatment groups was tested on all plates for each ELISA kit. The estimated repeatability within plates (98.35%,  $F_{16,17} = 0.898$ ) and between plates (98.21%,  $F_{16,17} = 0.770$ ) per kit was high. Antibody titre was calculated using the mean values (MV) of the measured optical density (OD) for the negative control (NC) and positive control (PC). The ratio sample to mean PC was then calculated using the following equation.

$$\text{S/P ratio} = \frac{\text{OD}_{\text{sample}} - \text{OD(MV)}_{\text{NC}}}{\text{OD(MV)}_{\text{PC}} - \text{OD(MV)}_{\text{NC}}}$$

Control individuals had antibody titres of  $0.039 \pm 0.123_{2SD}$  (*Salmonella* ELISA plate). Any serum S/P ratio of less than 0.1 was treated as a negative antibody response (within the confidence intervals of the control groups antibody titre values).

### **3.2.9 Statistics**

Before conducting analyses, normality of residuals and homogeneity of variance were checked. Raw values for antibody titres were log transformed to achieve normality. Data were analysed using linear mixed effect models (using the software “R”), in which female identity was included as a random factor. Binomial dependent variables were analysed using the statistical modelling package “lmer” using the family- binomial, for all other models I used the package “lme”. Factors affecting sex ratios of clutches were analysed using linear mixed effect models, binomial error and logit link function. In each analysis, the number of sons divided by the number of daughters plus sons was fitted as the response variable and the numbers of viable eggs and total clutch size in the brood was included as covariates in the model. Where sex ratio was the response variable models were weighted by clutch size to correct for variation in the size of clutch. Clutch 1 did not have enough data to produce maximal models therefore variables were explored independently. Initial male and paternal male effects across clutches were explored in separate maximal models and then a backward stepwise procedure was used to remove non-significant terms from the model.

Effect of female circulating antibody response was determined using the peak antibody titre per female and including this as a response variable in relation to egg traits and parental traits. Egg antibody levels pre and post incubation were also explored against egg traits. Furthermore a series of models were ran controlling for the effect of variation between mothers in their own antibody responses to challenge at specific points when eggs were laid, firstly by including these values in the model as a covariate along with day (to control for change in antibody level in the female over time). The relative level of circulating antibody transferred to the egg (egg antibody/ female antibody) was also calculated directly to investigate how this ratio related to other measures of a female’s antibody response as well as other female and male traits and egg traits. Total levels of antibodies were also explored either calculated as yolk mass\*yolk antibodies or egg mass\*yolk antibodies (further discussion in results).

### **3.3 Results**

#### **3.3.1 Dissection findings**

For this part of the analysis I was interested in determining at which point in the development of an oocyte female antibody transfer can be detected and at which point these correlate with female plasma levels. The relationship between the females' antibody levels just prior to culling and females developing oocytes were explored.

##### **3.3.1.1 At what stage of oocyte development are antibodies detectable?**

Antibody concentrations above the negative threshold were found in the follicles that were 2, 3 and 4 days prior to lay when concentrations were calculated from standardised samples corrected for overall yolk mass (oocyte 2 days prior to lay mean=1.022  $\pm$  0.16<sub>SE</sub>), oocyte 3 days prior to lay (mean=0.638  $\pm$  0.21<sub>SE</sub>) and oocyte 4 days prior to lay) (mean=0.195  $\pm$  0.06<sub>SE</sub>). The 4<sup>th</sup> oocyte (-5 days prior to lay), pooled samples and cluster had antibody levels below the negative threshold (day -5 oocytes; mean=0.042  $\pm$  0.03<sub>SE</sub>, pool1; mean=0.005  $\pm$  0.001<sub>SE</sub>, pool2; mean=0.001  $\pm$  0.001<sub>SE</sub> and cluster; mean=0.07  $\pm$  0.003<sub>SE</sub>) (Figure 3.1).

There was no significant difference between the mean level of antibodies found in the largest and next largest oocytes ( $q = 1.33$ ,  $p = 0.275$ ) but there was between the first and the third largest oocyte (-4 days) ( $q = 8.16$ ,  $p = 0.01$ ). Both the change between -4 to -3 and -3 to -2 days prior to lay oocytes was large. Percentage change in oocyte antibody levels (correcting for mass) was 245.65%  $\pm$  68.28<sub>SE</sub> for -4 to -3 days and 95.22%  $\pm$  28.66<sub>SE</sub> for -3 to -2 days. There was no difference between pool1 and pool2 and -5 day oocyte and oocyte -3 and -2 days ( $p < .05$ ).



**Figure 3.1** Antibodies were found in the 3 oocytes yolks closest to being laid when oocytes mass was accounted for, there was also a significant difference between oocyte- 4 and oocyte-2 days to be laid (indicated by \*\*). Negative threshold of detectable antibodies presented as a dotted line at 0.1 S/P value.

Not all studies that explore antibody concentrations take total yolk volume into account in their calculations so for comparison the analysis was repeated using the concentration in the standardised samples only. This analysis suggested detectable levels in the -2 days (mean= $0.743 \pm 0.089_{SE}$ ), -3 days (mean= $0.757 \pm 0.183_{SE}$ ), -4 days (mean= $0.713 \pm 0.135_{SE}$ ) and -5 days oocytes to be laid (mean= $0.309 \pm 0.17_{SE}$ ). Pooled samples and cluster antibody levels were below the negative threshold (pool1; mean= $0.074 \pm 0.01_{SE}$ , pool2; mean= $0.035 \pm 0.02_{SE}$  and cluster; mean= $0.042 \pm 0.02_{SE}$  (Figure 3.2). Comparing between the means (Tukey's multiple comparison test) of oocytes in the order in which they would be laid found a significant difference between cluster, pool1 and pool2 and -5 oocyte and oocyte -4, -3 and -2 days ( $p < .05$ ). Oocytes 4, -3 and -2 days were not significantly different from each other. Interestingly the greatest change between oocytes with detectable levels of antibodies (in concentration rather than absolute levels) was between oocytes -5 and -4 in line to be laid; mean percentage increase of  $149.92\% \pm 52.18_{SE}$  in contrast to the results found when only concentration was considered.



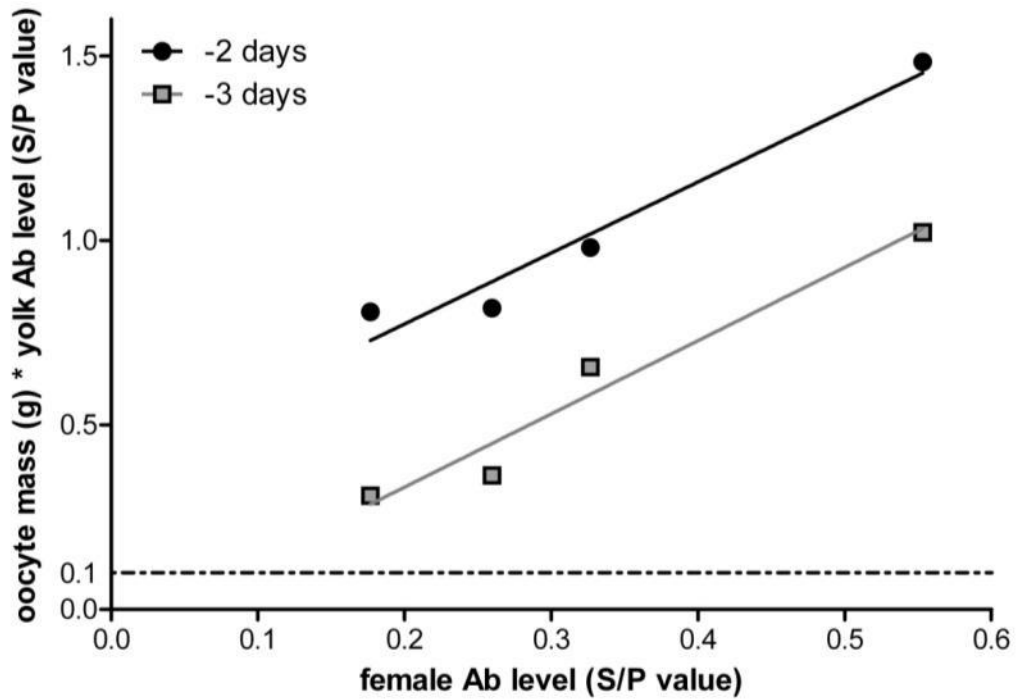


**Figure 3.2** Detectable levels of antibodies was found in the first 4 oocytes yolks (indicated by \*\*). Cluster, pool 2 and pool 1 had levels below the negative threshold of detectable antibodies (represented by the dotted line at 0.1 S/P value)

The difference between the two analyses suggest that all yolk components are not allocated consistently over time and demonstrates the importance of considering overall yolk volume when establishing total yolk concentrations of antibody.

### 3.3.1.2 Do oocyte antibody levels correlate with female antibody levels?

Antibody concentration in oocytes correlated with female antibody levels in the 1st and 2<sup>nd</sup> oocytes that will next be laid (-2 days to oocytes to be laid;  $r = 0.977$ ,  $p = 0.022$ ,  $n = 4$  and with -3 days to oocytes to be lay;  $r = 0.978$ ,  $p = 0.021$ ,  $n = 4$  (Figure 3.3). No significant correlation was found between female antibody level and oocyte antibody level in eggs later in sequence, suggesting that oocyte/yolk antibody levels only start to reflect antibody levels in the mother in the two days prior to it's fertilisation (Figure 3.3).



**Figure 3.3** There was a significant correlation between female antibody levels and oocyte antibody levels (correcting for mass) in oocytes -2 days to be lay (indicated by solid black line, solid circles and -3days oocyte and female antibody level (grey line, grey squares).

### 3.3.1.3 Dissection Summary

In summary, while low levels of antibodies may be present in the oocyte earlier in development, the main increase in antibody transfer is detectable 4 days prior to the egg being laid and fully correlates with female antibody levels from 3 days prior to being laid.

### **3.3.2 Do male traits influence female antibody levels?**

In this section I examine 1) how female blood antibody levels vary when paired with different males 2) how egg antibodies vary when females are paired to different males and 3) whether the relative allocation of female antibodies to the egg varies when females are paired with different males (control individuals were not included in this analysis).

#### ***3.3.2.1 Female blood antibody levels: Initial allocation decisions***

Female peak antibody titre did not differ between females initially paired with a large badge male compared to females initially paired with a small badge male (t-test;  $t = 0.30$ ,  $df = 5.45$ ,  $p = 0.774$ ). The time it took for a female to reach her peak antibody level, controlling for her baseline antibody titres, also did not differ between females initially paired with a large or small badge male;  $t_8 = -0.57$ ,  $p = 0.586$  (peak day also did not covary with peak levels produced;  $t_5 = -0.51$ ,  $p = 0.616$ ). Maternal antibody level across clutch one did not vary with male badge size ( $t_{82} = 0.70$ ,  $p = 0.491$ ), male mass ( $t_{14} = 1.44$ ,  $p = 0.152$ ) or male condition index ( $t_{14} = -1.70$ ,  $p = 0.109$ ).

#### ***3.3.2.2 Female blood antibody levels: response to pairing with different males***

Paternal male badge size did not influence female antibody levels (main effect;  $t_{195} = -1.2$ ,  $p = 0.207$ ) over the course of her reproductive life in this experiment. Male mass ( $t_{195} = -0.23$ ,  $p = 0.817$ ) and male condition ( $t_{195} = -1.17$ ,  $p = 0.240$ ) also did not influence the immune response of a female.

#### ***3.3.2.3 Female blood antibody levels: carry-over effects***

Initial male badge size had no carry over effect on female antibody level over the course of her subsequent clutches;  $t_{189} = 0.61$ ,  $p = 0.551$ . Initial male condition index ( $t_{14} = -1.30$ ,  $p = 0.194$ ) or mass ( $t_{14} = 0.47$ ,  $p = 0.637$ ) had no relationship with female antibody levels across clutches. Furthermore there was no relationship between initial male traits and the peak day of a females antibody response or any effect of

male badge size;  $t_6 = -0.48$ ,  $p = 0.762$  or condition index;  $t_6 = 1.94$ ,  $p = 0.089$  and mass;  $t_6 = 0.59$ ,  $p = 0.553$ ).

### **3.3.3 Do male traits influence the level of antibodies in the egg?**

#### **3.3.3.1 Level in eggs: initial allocation decision**

Male badge size in the first clutch did not affect the concentration of antibodies in yolks (pre incubation);  $t_6 = 1.64$ ,  $p = 0.347$ , or male condition;  $t = 1.64$ ,  $df = 6$ ,  $p = 0.347$ . There was also no relationship between the concentration of antibodies present and egg mass ( $t_{10} = -0.825$ ,  $p = 0.428$ ).

We then calculated total antibody content per egg by multiplying yolk antibody concentration by yolk mass. However, our yolk mass measurements were made 3 days into incubation and it is known via the results in Chapter 2 that embryos from females initially paired with large badge males have higher growth. It is therefore likely that these embryos have utilised more yolk. No difference was detected in the level of albumen in females initially paired to large badge males;  $t_6 = -1.68$ ,  $p = 0.118$  (mean albumen large badge  $= 1.64 \pm 0.08_{SE}$ , mean albumen small badge  $= 1.50 \pm 0.07_{SE}$ ). Therefore egg mass was also used to estimate total antibody content as yolk mass may have changed between 0 and 3 days of incubation. There was no effect of male badge size when total egg antibodies was calculated using yolk mass;  $t_6 = -0.06$ ,  $p = 0.960$ , however, a non-significant trend was found when exploring total antibody per egg calculated using egg mass;  $t_6 = 2.03$ ,  $p = 0.088$ .

#### **3.3.3.2 Level in eggs: when females are paired to different males**

Paternal male badge size, male mass and male condition all had no effect on the concentration of antibodies found in eggs produced by a females paired to different males over the course of four clutches: (badge size;  $t_{12} = 0.38$ ,  $p = 0.191$ , condition index;  $t_{21} = 1.38$ ,  $p = 0.181$ ). Egg mass, clutch size and embryo sex were also included in the model, the results of which are discussed later.

### **3.3.3.3 Level in eggs: carry-over effects**

Across clutches pre incubation antibody concentration in yolks were not significantly altered by the badge size of the male a female was initially paired with;  $t_4 = -0.41$ ,  $p = 0.699$ , initial males mean condition;  $t_4 = -0.33$ ,  $p = 0.793$ . Yolk mass (for eggs that didn't develop) did not covary with concentration of antibodies in them;  $t_{20} = 0.17$ ,  $p = 0.864$ .

The total antibody levels of the yolks, when calculated via yolk mass (egg antibody titre\*yolk mass) had no carry-over effect due to Initial male badge;  $t_4 = -1.21$ ,  $p = 0.291$  or an effect of paternal male badge;  $t_{12} = 1.63$ ,  $p = 0.126$ . When total antibody level was calculated with egg mass (egg antibody titre\*egg mass), initial male badge size has a significant effect on total levels; interaction between initial male badge size and clutch;  $t_4 = -2.73$ ,  $p = 0.009$ . This suggests initial male may have an effect on total antibody level at the point of lay, however this finding requires further investigation in unincubated eggs.

### **3.3.4 Relative level transferred: initial allocation decision**

Individual variation in the relative level of antibodies transferred in relation to maternal circulating levels has seldom been addressed but females may vary considerably in the relative amount of their own antibodies that they transfer to the egg (see Chapter 4). I therefore calculated the relative level of antibody transferred to eggs to examine whether this varied in relation to male traits. I controlled for female antibody response at the time each specific egg was produced and included these values as a covariate in the model, along with time (as a continuous variable). I also calculated the relative level transferred directly (female antibody level minus egg antibody level) to determine how relative levels of transfer may covary with other female traits. Table 3.1, presents these results across clutches.

#### **3.3.4.1 Relative level transferred: first clutch**

Within clutch one there was no effect of any of the male traits recorded on the relative amount of antibody transferred to offspring. Only female antibody level had a significant positive relationship with egg antibody concentration;  $t_{10} = 15.99$ ,  $p < .001$ . Furthermore, no relationship was seen between relative transfer and change in egg mass from clutch 0 to clutch 1 (no interaction between change and Initial male badge;  $t_5 = 0.57$ ,  $p = 0.581$  or egg mass change alone;  $t_6 = 1.89$ ,  $p = 0.869$ ). There was also no interaction with embryo sex on the relative level transferred.

Yolk mass at 3 days of incubation did not significantly differ between the females in these two initial pairing;  $t_7 = 1.55$ ,  $p = 0.225$ , contrary to what might be predicted due to the egg mass finding.

Total relative level of antibodies calculated using yolk mass had no relationship with male badge size;  $t_4 = 1.25$ ,  $p = 0.429$ . However, there was a non-significant trend in total relative level was calculated using egg mass;  $t_4 = 2.01$ ,  $p = 0.092$ .

**Table 3.1** Results from linear mixed effects analyses (across clutches). Maximal and minimal model values for female baseline, embryo sex, clutch size and egg mass shown are only for the maximal model including Initial male badge size. Values for other male traits and paternal male traits are presented from their maximal and minimal model, no different significant factors were found in these models.

<b>Model 1- Not controlling for female Ab levels</b>							
RESPONSE	EXPLANATORY	Maximal Model			Minimal Model		
		df	t	p	df	t	p
Yolk Ab	Initial male badge	4	0.05	0.622	4	-0.41	0.699
	Initial male condition	1	-0.33	0.793	1	-0.33	0.793
	Paternal male badge	7	1.88	0.100	12	0.38	0.191
	Paternal male condition	21	1.38	0.181	21	1.38	0.181
	Female baseline condition	16	0.07	0.989	18	0.04	0.964
	Embryo sex	16	0.16	0.869	19	0.21	0.834
	Clutch size	16	-1.86	0.119	22	-1.53	0.131
	Egg mass	16	0.61	0.546	19	0.28	0.778
	<b>Interactions</b>						
	Initial male badge* Embryo sex	16	-0.03	0.971	16	-0.03	0.971
	Initial male badge* Egg mass	16	-0.77	0.447	17	-0.79	0.437
	Paternal male badge* Embryo sex	7	-0.57	0.585	7	-0.57	0.585
	Paternal male badge* Egg mass	7	0.15	0.882	10	-0.22	0.828
	Embryo sex* Egg mass	16	0.61	0.544	16	0.61	0.544
<b>Model 2- Controlling for female Ab levels (by including in model)</b>							
RESPONSE	EXPLANATORY	Maximal Model			Minimal Model		
		df	t	p	df	t	p
Yolk Ab	<b>Female Ab</b>	14	4.57	<.001	21	9.46	<.0001
	Day	14	0.92	0.370	21	0.89	0.381
	Initial male badge	4	-0.02	0.979	4	-0.86	0.433
	Initial male condition	1	0.65	0.631	1	0.65	0.631
	Paternal male badge	7	-0.82	0.435	9	-0.11	0.908
	Paternal male condition	18	0.41	0.680	19	0.39	0.700
	Female baseline condition	14	1.03	0.317	16	0.30	0.765
	Embryo sex	14	0.16	0.873	19	0.32	0.749
	Clutch size	14	-0.59	0.560	20	-1.17	0.253
	Egg mass	14	-0.31	0.758	16	-0.42	0.679
	<b>Interactions</b>						
	Initial male badge* Embryo sex	14	-0.73	0.473	15	-0.77	0.452
	Initial male badge* Egg mass	14	-0.01	0.997	14	-0.01	0.997
	Paternal male badge* Embryo sex	7	-0.37	0.716	7	-0.37	0.716
	Paternal male badge* Egg mass	7	0.87	0.412	8	1.05	0.323
	Embryo sex* Egg mass	15	0.322	0.751	15	0.322	0.751
<b>Model 3- Controlling for female Ab levels (using calculated relative level transferred)</b>							
RESPONSE	EXPLANATORY	Maximal Model			Minimal Model		
		df	t	p	df	t	p
Relative yolk Ab	Initial male badge	4	0.66	0.541	4	-1.45	0.220
	Initial male condition	1	0.65	0.631	1	0.65	0.631
	Paternal male badge	7	0.01	0.996	11	0.42	0.675
	Paternal male condition	18	0.72	0.475	20	0.42	0.672
	Female baseline condition	15	-0.44	0.663	19	-0.63	0.531
	Embryo sex	15	2.05	0.058	22	1.35	0.190
	Clutch size	15	-0.06	0.947	17	-0.07	0.943
	Egg mass	15	0.04	0.967	18	-0.38	0.703
	<b>Interactions</b>						
	Initial male badge* Embryo sex	15	-1.31	0.209	16	-0.60	0.184
	Initial male badge* Egg mass	15	-0.63	0.532	15	-0.63	0.532
	Paternal male badge* Embryo sex	7	0.24	0.813	8	-0.10	0.919
	Paternal male badge* Egg mass	7	0.16	0.877	7	0.16	0.877
	Embryo sex* Egg mass	16	0.57	0.571	16	0.57	0.571

The impact of parental traits on the transfer of maternal antibodies

#### **3.3.4.2 Relative level transferred: response to different males**

Exploring the level of antibodies transferred to eggs correcting for female levels across breeding attempts there was no effect of male badge size, male mass or male condition on the relative amount transferred (Table 3.1, models 2 and 3).

Total relative level of antibodies calculated using yolk mass had no relationship with paternal male badge size;  $t_{14} = 0.626$ ,  $p = 0.542$ . This was also the case when total relative level was calculated using egg mass;  $t_{14} = 3.14$ ,  $p = 0.104$ .

#### **3.3.4.3 Relative level transferred: carry-over effects**

No carry-over effect of Initial male was found on relative transfer of antibodies (Table 3.1). Total relative level of antibodies calculated using yolk mass had no relationship with Initial male badge size;  $t_4 = -1.25$ ,  $p = 0.297$ . However, if relative total antibodies was explored (egg mass\* yolk Ab), female antibody concentration in the model resulted in a carry-over effect of initial male badge size interacting with clutch;  $t_4 = -3.46$ ,  $p = 0.001$ . Suggesting that the effect seen in total antibody levels being transferred is directly due to mass difference between eggs and not female antibody levels.

### **3.3.5 Embryo sex**

#### **3.3.5.1 Initial allocation of antibodies**

Within clutch one there was no relationship between embryo sex on the relative;  $t_6 = 0.868$ ,  $p = 0.418$  or concentration of antibodies transferred;  $t_6 = -1.56$ ,  $p = 0.169$ . Position in clutch also had no significant relationship with relative;  $t_6 = 2.16$ ,  $p = 0.074$  or concentration of antibodies transferred;  $t_5 = -1.15$ ,  $p = 0.302$ .

#### **3.3.5.2 Total level transferred**

The concentration of antibodies did not differ with embryo sex (Table 3.1).



Sons and daughters also did not vary in their total antibody level, yolk mass\*yolk antibodies;  $t_{23}= 0.31$ ,  $p= 0.763$  or as egg mass\*yolk antibodies;  $t_{23}= 0.27$ ,  $p= 0.782$ . Exploring position in clutch and embryo sex there was also no interaction between embryo sex and position in clutch on the total level of antibodies found, yolk mass\*yolk antibodies;  $t_{21}=-0.59$ ,  $p= 0.557$  or as egg mass\*yolk antibodies;  $t_{21}= -0.70$ ,  $p= 0.491$ . This was also true total antibodies was calculated using egg mass instead of yolk mass;  $t_{21}= -0.70$ ,  $p= 0.491$ .

### **3.3.6 Cost of immunity**

#### **3.3.6.1 Cost of immunity to female traits**

For this analysis, data from both control and treatment groups were considered. The difference between treatment groups at the beginning of the experiment (SalenvacT challenged and PBS control groups) did not differ in their body condition index;  $t= 0.35$ ,  $n=30$ ,  $p= 0.723$  (t-test). To determine if there was any affect of treatment over the experiment females mean condition per clutch were compared between treatments using a linear mixed effects model including female ID as a random effect. There was no difference found between control and treatment birds in female condition over the experiment ( $t_{36}= 0.01$ ,  $p= 0.990$ ). Clutch size also did not differ between treatments ( $t_{36}= -0.29$ ,  $p= 0.768$ ), or number of viable eggs ( $t_{16}= -0.221$ ,  $p= 0.827$ ) or sex ratio ( $z= 0.29$ ,  $p= 0.769$ ).

Within the SalenvacT groups female baseline condition had no relationship with the level of circulating antibodies a female had in the first clutch;  $t_{92}= 1.11$ ,  $p= 0.270$ , or clutch size;  $t_{92}= -1.40$ ,  $p= 0.163$ , suggesting no initial trade-off between condition and immunity. Only day had a positive significant relationship with female antibody levels in the first clutch and this is unsurprising as female were all at the beginning of their immune response and therefore in increasing stage of their antibody response;  $t_{92}= 5.93$ ,  $p= <.0001$ .

However, over the course of the experiment and all four clutches, there was a significant negative relationship found between female antibody levels (correcting for day) and clutch size;  $t_{179} = -3.35$ ,  $p < .001$ . Females with higher levels of antibodies produced smaller clutches. No significant relationship was found between female mean condition per clutch and her circulating antibody levels;  $t_{179} = 0.71$ ,  $p = 0.478$ .

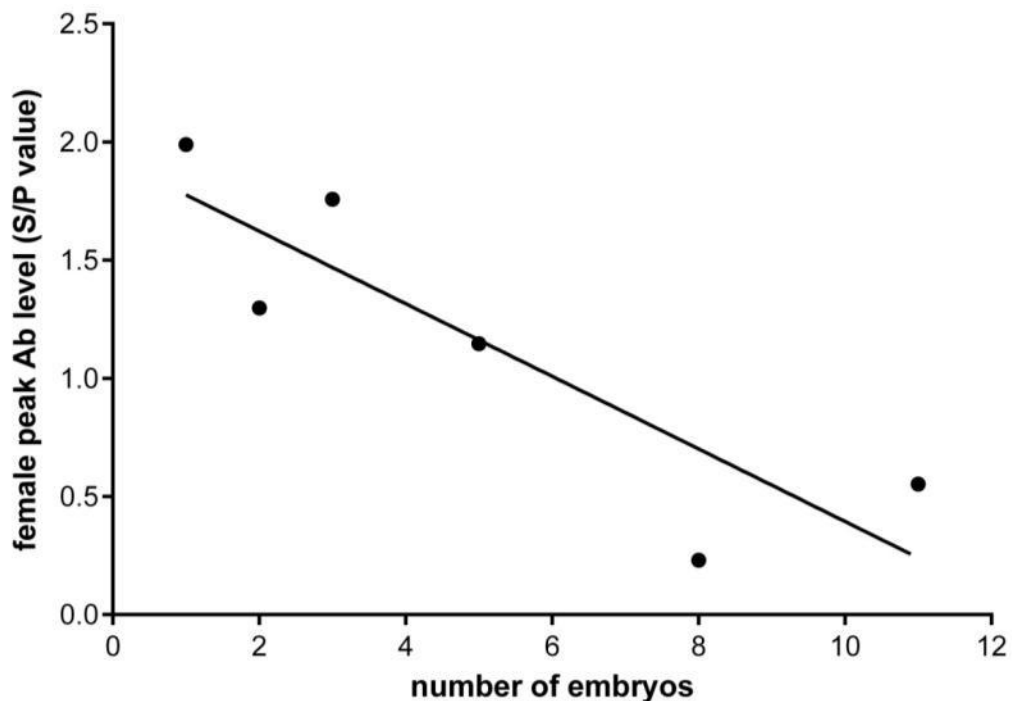
The effects of peak antibody level (as opposed to total antibody level described above) was also examined on reproductive traits. Peak antibody titre in mothers had no relationship with individual clutch size;  $F_{1,4} = 0.277$ ,  $t = -0.52$ ,  $p = 0.626$  or egg mass;  $t_4 = -0.71$ ,  $p = 0.51$ .

In eggs, pre incubation yolk Ab concentration had a no significant relationship with female mean condition;  $t_{51} = -1.63$ ,  $p = 0.118$ . There was also no relationship found between post incubation concentration ( $t_{51} = -0.65$ ,  $p = 0.519$ ) or relative levels ( $t_{51} = -0.64$ ,  $p = 0.523$ ) of Ab transferred in relation to female condition, or total level transferred in eggs  $t_{51} = -0.51$ ,  $p = 0.614$ . None of these values have any relationship with female baseline condition index; all  $p > 0.4$ .

### **3.3.6.2 Cost in relation to offspring traits**

In the previous chapter I found that viability within a clutch was significantly affected by paternal male badge size, clutch size and egg mass. Therefore, a model including these variables plus female peak antibody titre (for SalenvacT treated individuals) was run to explore their effects on viability. Like the findings in the previous chapter there was a significant effect of paternal male badge size (larger badge males produce more viable eggs) on viability but this was independent of egg mass;  $z = 2.04$ ,  $p = 0.041$ , clutch size ( $z = 2.21$ ,  $p = 0.027$ ) was retained in the model. Egg mass was retained in the model (due to AIC) however was non-significant ( $z = -1.23$ ,  $p = 0.217$ ). Female peak titre ( $z = 2.10$ ,  $p = 0.035$ ) was also found to be associated with the number of viable embryos produced across all 4 clutches with higher peak titres being associated with lower viability.

To determine the overall effect of the antibody response over the whole experiment all eggs and total number of embryos (across whole experiment) was explored. No significant relationship was found between the number of eggs produced and peak antibody response;  $t_{14} = -1.687$ ,  $p = 0.117$ . There was also no significant difference between treated and control individuals in overall number of eggs produced, although there was a non-significant negative trend of treatment;  $t_{25} = 4.08$ ,  $p = 0.072$ . However, female peak antibody response had a significant negative relationship with number of embryos produced ( $t_4 = -3.55$ ,  $p = 0.023$ ), suggesting a trade-off between viability and high levels production of antibodies (Figure. 3.4).



**Figure 3.4** Female peak antibody level over the cause of her antibody response in relation to number of embryos she produced, negative relationship shown;  $t_4 = -3.55$ ,  $p = 0.023$ .

Yolk Ab post incubation had a significant relationship with pre incubation yolk Ab;  $t = 9.447$ ,  $p < .0001$ ,  $n = 6$  (30 eggs). However, relative antibody titres had no relationship with female antibody titres;  $t_{23} = 0.58$ ,  $p = 0.561$ . Furthermore mean

relative transfer over a clutch had no relationship with female peak antibody titre;  $t_5 = -0.38$ ,  $p = 0.719$ .

It is also unlikely that antibody transfer inhibits development of embryos as there was no difference in the level of antibodies transferred found between developed and undeveloped eggs pre incubation;  $t_{47} = -0.811$ ,  $p = 0.422$  or relative levels;  $t_{47} = -0.471$ ,  $p = 0.639$  suggesting that the proportion or concentration transferred did not impact on viability.

### **3.4 Discussion**

In the light of recent findings and my own work from Chapter 2 I examined whether females adjust their allocation of maternal antibodies in response to male traits. I found no evidence that females' own antibody responses varied in relation to male traits or that females actively allocated more antibodies to eggs in relation to male traits. In Chapter 2 I found that females laid larger eggs when initially paired with large badged males and that this effect on egg size carried over to subsequent clutches when paired with different males. I found evidence to suggest this may result in a greater total antibody concentration in eggs as a result but this needs to be verified by looking at antibody levels in unincubated eggs. I found no evidence to suggest a sex bias in allocation and no difference in the utilisation or decay of maternal antibodies by the two sexes between initial titres pre incubation and antibody levels at 3 days of incubation. Evidence was found to suggest that producing an antibody response and the level to which this response occurs (in the absence of a pathogen) may trade off with both the quantity and viability of eggs a female can produce across a number of breeding attempts.

#### **3.4.1 Developing yolk findings**

To my knowledge only one paper discusses the transfer of antibodies from mother to developing oocytes in detail; examining the average level of transfer of antibodies in oocytes from white Leghorn chicken across development stages (Kowalczyk et al., 1985). The author found that 8 to 3 days before oocyte maturity mass and antibody content increases exponentially and that the maximum uptake of antibodies occurred 3 to 2 days before oocyte maturity. Here I show that maternal circulating antibody titres have a significant relationship with oocyte/yolk antibody levels, however this relationship is only found at specifically the two latest points of development of the oocyte where the yolk is well formed, illustrating that only in the later parts of yolk development antibodies are transferred at a high levels and reflect levels present in the mother. As expected, oocyte antibody levels and oocyte mass have a significant relationship with time to laid. However, one of the most interesting findings from the experiment was that there was a specific point in the development of the oocytes

where the difference between oocyte and previous oocytes mass and antibody titre differed significantly. This was between oocytes 4 and 3, suggesting that at this point there is a surge in antibodies into the oocyte and this may also be the case for other yolk components such as hormones (Groothuis and Schwabl, 2008). The findings from this part of the experiment suggests that levels found in a egg correlate with antibody levels in the mother 24hrs prior to lay and may even correlate with a mothers antibody levels at 48hrs prior to lay.

The relative timing of this event is important to consider in relation to how interactions with different males could influence allocation decisions. Eggs are laid 24 hours after fertilization and maternal deposition of antibodies and other major yolk components commences a further 3 days prior to fertilisation. If males were to influence antibody levels directly then interactions would have to take place several days prior to laying when the egg is formed and total IgY antibody allocation to the yolk would be complete 24 hours prior to lay. Addition of other constituents could however influence the concentration of yolk antibodies right up to the point the eggs is laid. These results demonstrate the importance of considering egg or yolk volume when measuring allocation effects as simply considering concentration levels of a set aliquot size may not reflect the same patterns.

### **3.4.2 Female antibody response in response to male traits.**

Differences in female allocation of antibodies when paired to a particular male may occur via a number of mechanisms, firstly, females may alter their immune response in relation to the male they are paired with, secondly, females may alter egg traits with subsequent impact on maternal antibody levels (e.g. by altering egg mass), or finally, females may increase the relative level of antibodies transferred to eggs.

No evidence was found to suggest that any aspect of the female immune response was affected by the male characteristics I examined. While limited studies have explored maternal immune response and partners traits this was also the case in another study exploring antibody production and transfer to offspring, where

maternal levels had no relationship with male traits (Saino et al., 2002b). In this study there appears to be no cost of the transfer level of antibodies on maternal condition, however the immune response of the mother itself may be costly as it had a negative relationship with the viability of eggs and the total number of viable embryos produced by females across her reproductive life within the experiment.

### **3.4.3 Yolk antibody levels in response to male traits.**

Differential allocation suggests that females may alter allocation or resource to offspring when partnered with preferred males. In chapter 2 I found that females altered the number of eggs they produced when paired with different males, however, in this study I found no effect of paternal badge size on maternal antibody transfer to yolks. This was the case for all three measure of yolk Ab used (pre incubation, post 3 days incubation and relative transfer of Ab). This is similar to a study in collard flycatchers which found no effect of male secondary sexual traits (forehead patch size) on yolk antibody levels (Hargitai et al., 2006).

In the previous chapter I found a significant effect of badge size on egg mass as a consequence of a female's initial allocation decisions when they first paired carrying-over to subsequent breeding attempts. In this chapter I found no carry over effect on yolk antibody concentration or on our initial estimate of total antibody level. However yolk antibody level was initially calculated using the remaining yolk mass after 72 hours of incubation and our findings from chapter 2 suggest utilisation of yolk may have differed during this time (initial carry over effect on embryo mass). When I used the change in egg size to calculate the total antibodies transferred per egg (calculated as egg mass\*antibody level), there was a significant carry-over effect of a female's initial allocation decision on antibody level across clutches. Therefore for this part of the experiment there appears to be some change between 0 and 3days of incubation that we could not observe but is important to understanding how the carry-over effect seen on egg mass influences total antibody levels. Does yolk mass reflect egg mass with relation to carry-over effect and this is just not seen at 3 days as the embryo has already absorbed some of the yolk? Or do other components of the egg vary in relation to carry-over for example albumen mass as this too is an

important component for embryo development (Bonisoli-Alquati et al., 2007)? In this study the albumen was not larger for females initially paired with a large badge male compared to females who were initially paired with a small badge male. Only further study can pull these apart to determine how total maternal antibodies are effect when a carry-over effect is present.

#### **3.4.4 Maternal transfer of antibodies to embryos of different sexes**

Sex differences in allocation of maternally transferred substances have been found (Saino et al., 2003, Martyka et al., 2011) as well as other components important to physiological development such as androgens (Groothuis et al., 2005), corticosteroid (Love et al., 2005) and carotenoids (McGraw et al., 2005). Therefore a key part of this chapter was to determine if offspring of different sexes are allocated maternal antibodies differently or if there is a physiological process causing increased decay of antibodies in one sex. I found no difference in the level of antibodies allocated to male and female offspring and no significant difference between pre incubation levels of antibodies and post incubation levels between male and female offspring. This suggests that in my population there is no variation in decay or assimilation of yolk antibodies between offspring sex (up to 3 days of incubation).

#### **3.4.5 Cost of immunity**

As expected the concentration of yolk Ab pre incubation was positively correlated to yolk Ab post incubation and both were correlated with maternal plasma Ab, in line other literature (Grindstaff et al., 2005, Blount et al., 2002, Martyka et al., 2011). However, relative transfer was not correlated with female peak titres, suggesting that females who produce high circulating antibody levels may not necessarily transfer relatively more antibodies to their eggs than other individuals (see Chapter 4).

Hargitai et al., 2006 found a non-significant positive relationship between body condition and female circulating Ab levels and suggest that high antibody titres could indicate high-quality females. As suggested by other studies up-regulation of the immune system may be costly (Lochmiller and Deerenberg, 2000). Therefore if



circulating levels of antibodies in the mother reflect the amount transferred to eggs we may find only good quality individuals can produce and transfer high levels of antibodies.

In this chapter I also found that female antibody response had a significant negative effect on egg development. Females with a greater peak in circulating antibodies in her plasma produced a smaller number of viable eggs over the course of the experiment. Furthermore when I explored the levels of circulating antibodies on specific days across a clutch I detected a negative relationship with clutch size, suggesting that exploring peak titres of a females antibody response only picks up larger effects on reproduction, however with more resolution (exploring levels day by day) the antibody response is not only affecting viability but also clutch size itself. Other studies have found in females who are responding to an immune response reproduction is compromised. For example in pied flycatchers (Ilmonen et al., 2000) scientists have found females responding to diphtheria-tetanus vaccine (in one study site) had a reduction in fledging success compared to control individuals. This study adds to the body of literature showing a trade off between immunity and reproduction and highlights these effects are detectable during the earliest stages of development before hatching even occurs (for review see, Lochmiller and Deerenberg, 2000).

### **3.4.6 Summary**

In this study I add to the growing field of study exploring differential allocation and maternally transferred immunity. In this study there was no variation found in the transfer of maternal antibodies or maternal antibody response of mothers due to paternal traits. This highlights that maternal antibody transfer and maternal antibody response in this species are not traits affected by male traits. However, I again highlight that carry-over effect of initial allocation decisions may impact on antibody levels available to developing offspring despite no direct effect of these interactions on female antibody production. The effect of female antibody response on viability and clutch size but no effect on other reproductive traits suggests that there may be a trade-off between the level of an antibody response and the quantity of eggs

produced. This may become a highly important trade-off in wild systems with females under low resources and high parasite burdens.

# CHAPTER 4

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## **Individual variation in the transfer of maternal antibodies to the embryonic environment**

### **4.1 Introduction**

The immune response plays a key role in protecting individuals from disease and this protection can be extended to offspring via the transfer of maternal immunity (for review see, Hasselquist and Nilsson, 2009). Maternal immunity can be crucial in protecting offspring in early life when their own immune system is developing and hence can play an important role in health and fitness. Maternal immunity is induced when females are exposed to a pathogen in the maternal environment prior to or during reproduction but can also be induced by health interventions such as vaccination. Maternal antibodies are a major part of this response and their transfer to offspring is therefore an important source of phenotypic variation potentially affecting the ability of offspring to deal with infection. Such maternal effects, whereby female allocation during pregnancy or egg production influences the phenotype of developing young, can have a major impact on the relationship between genotype and phenotype. They can therefore be a powerful evolutionary force that can adjust the speed of trait change in response selection of both host and pathogen (Mousseau and Fox, 1998b) (Kirkpatrick and Lande, 1989) as well as having a direct impact on offspring health.

Factors that affect an individual's immune response have been well studied; in contrast less is known about the factors determining the level of maternal antibody transfer to offspring. Despite being a widespread mechanism playing a role in offspring protection from disease across taxa, little is known, for instance, about how

females may vary in their levels of antibody transfer or how antibody transfer may change over time within the same individual; and how females respond to different types of infection and the consequence for offspring produced. It has been assumed that maternal antibody transfer is passive with levels in offspring simply reflecting levels of antibody in the mother at the time of egg production (Kowalczyk et al., 1985); in birds, for example, much of the early literature from poultry science suggests that the level of antibody transfer is fairly invariant and that females consistently transfer approximately 20% of their circulating antibody level to each egg produced (Brambell, 1970). However, variation at the individual level has seldom been fully explored and whether variation may exist between individuals in the proportion of circulating antibodies transferred to offspring remains unclear. If variation exists for this trait, then the level of transfer may itself be a trait open to selection or manipulation if Ab levels are indeed related to offspring survival (Boulinier and Staszewski, 2008)

Recent literature from the field of evolutionary ecology has suggested that maternal responses to infection may be less invariant than previously thought. Levels of immune transfer may be allocated differently in response to a number of factors including mating partner (Saino et al., 2003), sex of offspring (Martyka et al., 2011) or position in a clutch (Blount et al., 2002). This is consistent with life history theory, which predicts that the level of antibody transferred to offspring should vary, depending on the likelihood of their success. However, it is unclear whether the degree of antibody transfer is actively adjusted for different offspring or whether the levels transferred are simply a consequence of changing levels of maternal antibodies as different eggs in a laying sequence are formed.

Establishing how patterns of antibody allocation relate to a female's antibody production within and between individuals is necessary to establish the ecological and evolutionary implications of these maternal effects. However, progress has been hampered by a lack of information on how antibody levels change in mothers during the period when eggs are being formed. Data from the general immunological literature would suggest the typical pattern of an antibody response post-challenge is

characterised by a slow then increasing rise to peak antibody level, followed by a steady decline as antibodies are catabolised (Cruse and Lewis, 2004). Post acute infection, cells produced by the primary immune response persist as memory cells allowing rapid production of further antibodies to be produced if there is re-infection. Maternal transfer would therefore be expected to track this pattern of antibody production over time. However, co-infection is common with females potentially producing a range of specific antibodies to a range of different types of pathogen they encounter in the environment (Petney and Andrews, 1998, Cox, 2001). As a result, the response to one immunological challenge may be modulated by current or historical immune responses as individuals are often exposed to multiple challenges over a short period of time (Martin et al., 2006). In this case, activation of one component of the immune system may limit or promote the expression of another – bacterial challenge for example may stimulate the immune response in ways that make responses to a viral challenge initiate more quickly. Furthermore, individuals may vary in their ability to respond to these different types of challenge due to genetic and ontogenetic differences in immune development (Ardia et al., 2011) – how these differences then impact on maternal transfer has yet to be examined

Here, I experimentally investigated how female Chinese painted quail, *Coturnix chinensis*, vary in their own immune response and the level at which this is passed to the egg over the course of an antigen challenge. I also tested the response of females to different types of challenge (a bacterial and viral challenge) and investigated variation in the level of antibody transferred by females. Further to this I measured a number of female traits and analysed their relationship to the transfer of antibodies. I then considered the evolutionary and ecological implications of maternal antibody allocation and addressed how observed patterns of antibody allocation over the course of an extended clutch might explain differences in allocation for different offspring.

## **4.2 Method**

General husbandry conditions were identical to those outlined in Chapter 2 and 3 and therefore only briefly outlined here.

### **4.2.1 Study system: Establishing the maternal generation**

The study was conducted using a colony of Chinese painted quail (*Coturnix chinensis*), established at the University of Edinburgh, to provide parental birds which had no previous exposure to common pathogens. To establish the parental generation, eggs were collected from multiple commercial breeders around the UK and sprayed with Ambicide<sup>TM</sup> (1% dilution) prior to incubation to prevent any transfer of common environmental pathogens into the colony. Eggs were incubated under standardised conditions (37-38 °C and 40-50% humidity rising to 70% prior to hatching) then brooded for 24 hours prior to transfer to communal cages. Chicks were fed *ad libitum* and kept in large (2,400 x 500 x 375 mm) mixed sex cages (14 birds per cage) until they reached sexual maturity. Cages contained wood shavings, multiple feeding stations, covered areas and sand baths, a setup which allows birds to follow their full repertoire of natural behaviour. Birds were ringed to allow individual identification at six weeks of age. Standard biosecurity measures to maintain a pathogen free flock were in place throughout the duration of colony establishment and the experiment.

### **4.2.2 Experimental protocols**

Two weeks prior to the start of the experiment, birds were moved into breeding groups in which they remained for the duration of the experiment. Birds were housed in cages (800 x 500 x 375 mm) in a ratio of three females to one male. Each cage was lined with wood shavings, and contained a nest area for each female and a communal sand bath. Adult birds were maintained on a photoperiod of 16 h:8 h L:D and on a diet of Haith's finch seed, EMP, Prosecto Insectivorous and oystershell in a mix of 20% protein, 2.5% calcium and 77.5% seed for the duration of the experiment. Total feed per cage was 71.6g based on 17.9g of feed per bird, which is 10% less than adult laying female *ad lib* consumption over a 24 hour period (unpublished data).

*C. chinensis* eggs are highly polymorphic in both background colour and in the presence/absence and type of markings. Once laying commenced, females were observed continuously to identify the colour morph of each female enabling eggs to be assigned to individual females. Birds were housed so that all females in a cage laid sufficiently different eggs to allow eggs to be confidently assigned to a given female. Eggs were removed on a daily basis to allow females to continue laying over the course of the experiment. Biometrics of females (total body mass (g) and tarsus length (mm)) were measured and baseline blood samples were collected on day 0 prior to treatment, along with tarsus and mass measurements.

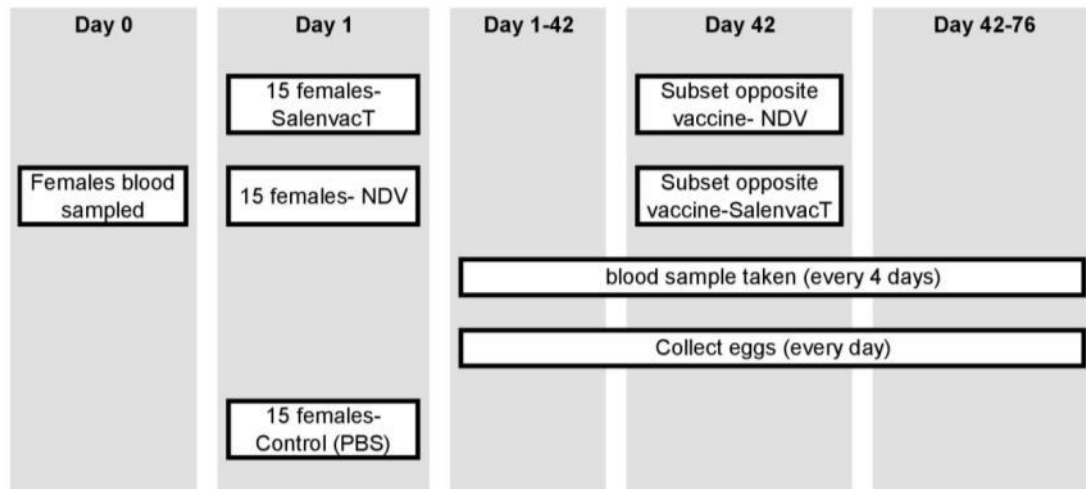
#### **4.2.3 Challenge of treatment groups**

In each cage, females were randomly assigned to one of three treatment groups (Image 4.1): in the negative control group females were injected with 0.1ml of phosphate-buffered saline (PBS) (n=18), bacterial challenge group females were challenged with 0.1ml inactivated *Salmonella* vaccine (SalenvacT, Intervet) (n=19) and viral challenge group females were challenged with 0.25ml inactive Newcastle Disease virus (NDV) (Paramyxo P201 Intervet) (n=18). Each cage therefore contained one bird from each treatment in order to control for cage effects. A control group was included in the experiment to compare the effect of vaccination on female condition and egg production. Blood samples were taken immediately prior to challenge then every four days up until 39 days post-vaccination. Samples were collected by puncturing the metatarsal vein using a sterile needle and taking up blood in a 100µl capillary tube. Blood samples were centrifuged on day of collection at 9050 rcf/g for 10 minutes and serum removed and stored at -20°C until antibody analyses were conducted. All work was conducted under Home Office License No PPL 60-4115, with full ethical approval from the University's ethical committee and with veterinary supervision throughout.

#### **4.2.4 Repeatability of response over different treatments**

A subset of individuals was re-vaccinated at 42 days post first treatment. Salenvac T individuals were vaccinated with 0.25ml NDV at day 42 (n=4) and NDV individuals

were revaccinated with 0.1 ml SalenvacT (n=3). Blood samples were collected at four-day intervals and eggs were collected daily from these birds until 76 days post-first vaccination (Image 4.1).



**Image 4.1** Flow diagram of experimental protocol for first challenge (day 1) and second challenge (day 42) for a subset of females.

#### **4.2.5 Measurements of maternal traits**

The mass and condition of all females were measured on all blood-sampling days. Pectoral muscle and fat were assessed using BTO guidelines on scoring size (Ringers' Manual BTO, Thetford). Pectoral muscle was scored 0 if the sternum was sharp and muscle depressed, 1 if the sternum was still distinguishable but not sharp and 2 if the muscle was rounded over the sternum. Fat levels were scored 0 if no fat was visible in tracheal pit, 1 if there was a trace of fat and 2 if the tracheal pit was obscured by fat. Because all birds in this experiment were kept under standardized conditions, body condition was calculated by  $\text{mass} / \text{tarsus length}^3$ , though different measures of condition revealed qualitatively similar results (see, Galvan, 2010, for discussion). Eggs were generally laid in the early afternoon and collected on a daily basis throughout the experiment.

#### **4.2.6 Egg measurements and antibody extraction**

Egg length, width and mass were recorded at the time of collection, after which eggs were stored at -20°C. Antibody extraction and analysis and yolk mass calculation were performed on defrosted samples. For each egg, egg volume (V) was calculated



using the volume coefficient ( $K_v$ ) calculated for *Coturnix* eggs (Hoyt, 1979):

$$V = K_v \cdot L \cdot B^2$$

Length (L) and breadth (B) are the maximal dimensions of specific eggs and  $K_v = 0.51$ .

A female's total investment in eggs was calculated as the sum of V across all a female's eggs over the course of the experiment:

$$\sum V$$

Antibodies were extracted from egg yolk following the methods of Mohammed et al. 1986 . The homogenized yolk was diluted 2:1 in phosphate- buffered saline (PBS) and vortexed for 2 minutes. Chloroform was added to the egg yolk/ PBS mixture at a 1:1 ratio, and vortexed for a further 2 minutes. The mixture was then centrifuged on 9050 rcf/g for 10 minutes, resulting in separation of the mixture into three layers; a top layer containing PBS and supernatant (used for antibody analysis), a deposit of fatty lipids in the middle layer and an organic phase containing chloroform and carotenoids in the bottom layer. The top layer was removed and stored at minus 20 until samples could be run.

#### **4.2.7 Antibody analysis: Enzyme-linked immunosorbent assay (ELISA)**

Specific enzyme-linked immunosorbent assays (ELISAs) were performed for treatment and control groups. For the SalenvacT treated individuals a *Salmonella*-specific ELISA test was performed using FLOCKTYPE<sup>®</sup> *Salmonella* ELISA kit (Labor Diagnostik Leipzig, Germany). For the Paramyxo P201 treated individuals, a Newcastle disease virus-specific ELISA test was performed using FLOCKTYPE<sup>®</sup> recNDV ELISA kit (Labor Diagnostik Leipzig, Germany). Both kits were manufactured for chicken serum and plasma but quail antibodies are also detected and a number of studies have used anti chicken antibody to detect antibodies in various quail species. Previous pilot work had established the appropriate dilutions to ensure antibodies lay within the bounds of the detectable range of the ELISA kits and dilution curves confirmed these lay within the linear part of the test.

Yolk extractions were diluted 1:62 and blood samples were diluted 1:124 with the buffer provided. The optical density (OD) of the resulting solution was read in a spectrophotometer at 450 nm immediately after stopping the reaction, and corrected for using positive and negative controls supplied in the kit. To estimate the repeatability of the method, one sample for each of the treatment groups was tested on all of plates for each ELISA kit. The estimated repeatability within plates (98.35%,  $F_{16,17} = 0.898$ ) and between plates (98.21%,  $F_{16,17} = 0.770$ ) per kit was high. Antibody titre was calculated using the mean values (MV) of the measured optical density (OD) for the negative control (NC) and positive control (PC). The ratio sample to mean PC was then calculated using the following equation.

$$\text{S/P ratio} = \frac{\text{OD}_{\text{sample}} - \text{OD(MV)}_{\text{NC}}}{\text{OD(MV)}_{\text{PC}} - \text{OD(MV)}_{\text{NC}}}$$

Control individuals had antibody titres of  $0.0491 \pm 0.0447_{2\text{SD}}$  (*Salmonella* ELISA plate) and  $0.029 \pm 0.067_{2\text{SD}}$  (NDV ELISA plate). Any serum S/P ratio of less than 0.1 was treated as a negative antibody response resulting in 9 birds (1 SalenvacT, 8 NDV) being excluded from the study. Seven birds (4 SalenvacT, 3 NDV) did not produce a full sequence of eggs or ate their eggs before they could be collected and were also excluded from the study. I was therefore able to collect a full sequence of blood and egg antibody levels over the course of a laying sequence for 12 control females, 14 bacterial challenge females and 7 viral challenge females.

#### **4.2.8 Statistical analyses**

Statistical analyses were performed in R (version 2.10.1). I fitted mixed models using the lme4 package. I investigated the effects of a range of factors on the amount of antibody transferred to eggs using linear mixed effects models and linear models and calculated repeatability estimates from the intraclass correlation coefficient. Treating egg antibody level as a dependent variable, I investigated the effects of treatment, females' circulating antibody level, yolk mass and number of days since challenge on the amount of antibody transferred to the embryonic environment. Female ID was included in all repeated measures models as a random effect. Blood samples were collected from females every 4 days. To examine how egg antibody levels varied with a female's antibodies over the course of the antibody response I ran a smoothing spline on the female's antibody response to get predicted values for days where blood samples were not collected. Lambda was set at 0.1 to maximise a close fit to the data. The effects of maternal body condition index, fat score, muscle score, yolk mass and total egg investment were also examined. Body condition index, yolk mass, total egg investment and female circulating antibody level were included as continuous variables and treatment (either SalenvacT or NDV), fat score, muscle score and day sampled were treated as categorical. A backward stepwise procedure was used such that non-significant terms were sequentially removed from the model.

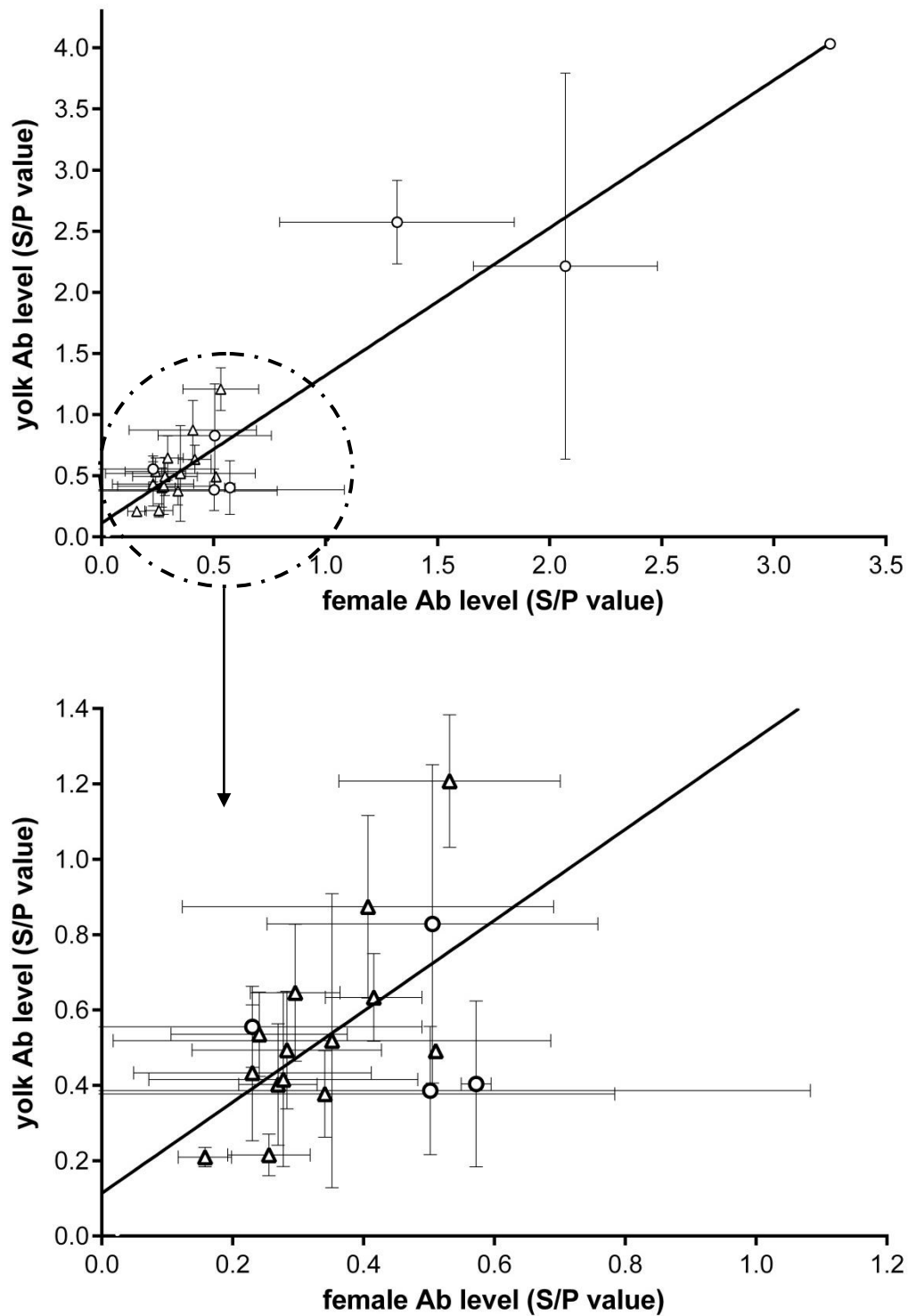
I also ran a series of models controlling for the effect of variation between mothers in their own antibody responses to challenge by calculating the ratio of circulating antibody transferred to the egg (egg antibody/ female antibody) and using this as my dependent variable. This is a measure of the proportion of antibodies a female transfers, regardless of her own response. This allowed us to investigate how this ratio related to other measures of a female's antibody response such as peak titre and peak day as well as other female traits such as body condition index, fat, muscle and egg investment.

### 4.3 Results

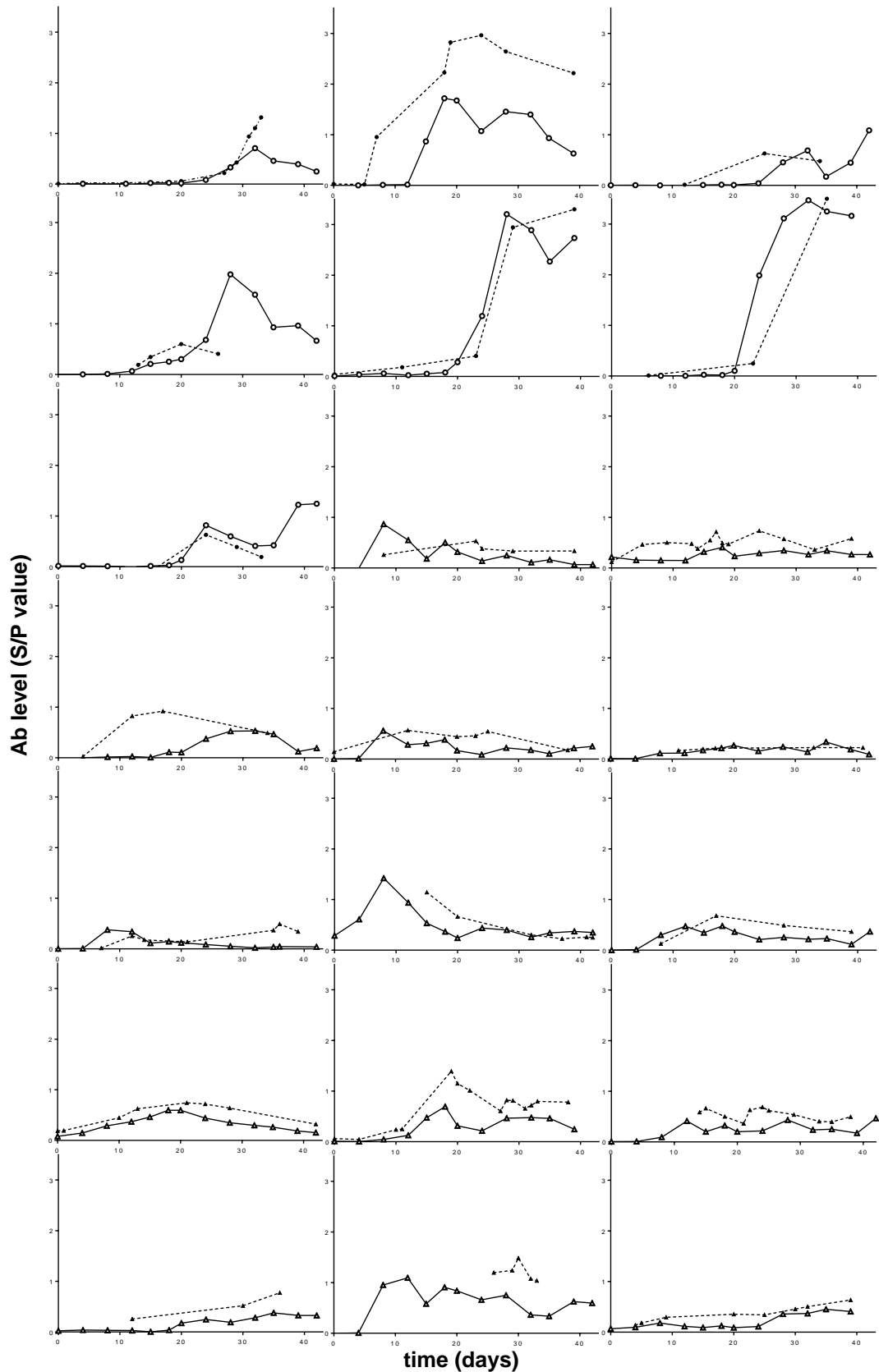
Within the NDV and SalenvacT individuals mean female antibody levels were generally found to have a positive relationship with mean egg antibody levels (Figure 4.1). However, there was considerable variation between females in their pattern of antibody (Ab) response over the laying period and in the amount of antibody they transferred to their offspring. Females showed a steady increase in antibody level in the days post challenge until peak antibody level was reached, followed by a slow decline in antibody level over time (Figure 4.2). Females varied both in the time they took to reach their peak of circulating antibody level (mean peak day  $22.22 \pm 3.425_{SE}$ ,  $n=21$ ) and in the magnitude of their response (mean peak size  $1.127 \pm 0.163_{SE}$ ) (Figure 4.2). The number of days post challenge therefore had a large effect on the level of antibody present in the eggs laid at the corresponding time ( $F_{34,99} = 2.29$ ,  $p = 0.006$ , Table 4.1).

**Table 4.1** Maximal and minimal linear mixed effects model of egg antibody level (S/P value), significant effects highlighted in bold.

RESPONSE	EXPLANATORY	Maximal Model			Minimal Model		
		df	F	p	df	F	p
Yolk Ab	<b>Female Ab</b>	17	129.75	<.0001	76	128.85	<b>&lt;.0001</b>
	Yolk mass	17	1.71	0.207	18	0.85	0.362
	Female mean condition	17	2.24	0.152	19	1.14	0.310
	Fat score	10	3.22	0.102	19	0.47	0.641
	Muscle score	10	0.001	0.979	11	0.09	0.917
	Egg investment	10	0.101	0.756	12	0.26	0.619
	Female start Ab	10	0.005	0.94	10	0.01	0.940
	Female peak Ab	10	0.54	0.478	13	0.18	0.676
	Treatment	10	0.601	0.455	14	0.25	0.624
	<b>Time (day)</b>	17	2.82	0.111	76	2.26	<b>0.007</b>
	<b>Interaction</b>						
	Female mean condition* Yolk mass	17	2.72	0.117	17	2.72	0.117
<b>Random Effects</b>							
	<b>Variance component</b>						
Female ID		0.1					
Residuals		0.04					

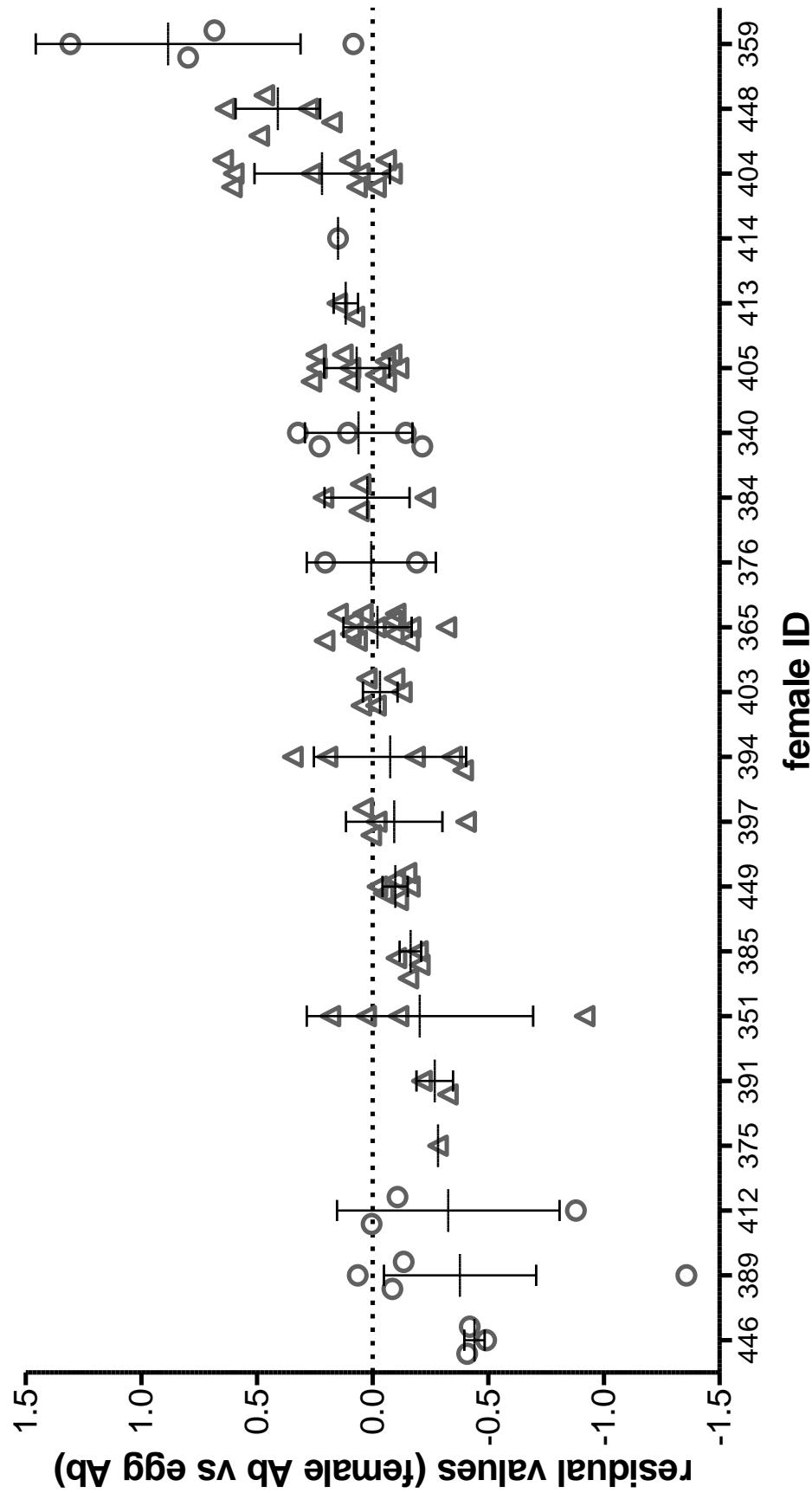


**Figure 4.1** Positive relationship between females' mean circulating and mean egg antibody level (Ab);  $F_{1,20} = 122.257$ ,  $P < .001$ . Horizontal error bars show 1 standard deviation above and below female mean antibody level and horizontal error bars show 1 standard deviation around mean egg antibody levels.



**Figure 4.2** Individual female and egg antibody levels graphed over time for n=21 females. Solid line and open points indicate female antibody level (triangles- NDV vaccinated, circles- SalenvacT vaccinated). Dotted line and solid points indicate egg antibody level (triangles – NDV vaccinated, circles- SalenvacT vaccinated).

After controlling for differences due to sampling time (Egg Ab ~ Day sampled + treatment + Female (random)), I found significant variation in the absolute amounts of antibody transferred to eggs (log likelihood ratio between a model including female ID or not;  $X^2_1 = 31.87$ ,  $p < .001$ ) (treatment effect;  $t = 2.69$ ,  $p = 0.016$ ). To examine what proportion of a female's own antibody response was transferred I controlled for differences in a female's own circulating level of blood antibodies by adding female circulating level to the model (Egg Ab ~ Female Ab level + Day + Treatment). Again I found considerable variation in the relative amount of circulating antibody transferred to the egg (log likelihood ratio between a model including female ID or not (original model Table 4.1);  $X^2_1 = 18.412$ ,  $p < 0.001$ ). Within females, on the other hand, the repeatability of the relative amount transferred over the course of the laying sequence was high (intraclass correlation coefficient;  $r = 0.714$  (Figure 4.3) and this ratio remained consistent when time of sampling was not included in the model;  $r = 0.727$ ). Assuming a female's blood volume equals 10% of her body weight (Fudge, 2000) my results suggest that females transfer on average 25.5% of their circulating antibody level to each egg which is, in line with previous findings (Kowalczyk et al., 1985). However, the variation between females was high with the lowest responders transferring an estimated 9.21% (range 7-10%) and the highest transferring 38.4% (range 22-58%).



**Figure 4.3** Inter-individual variability in the capacity to transfer antibodies. Relationship between residual of the relationship between female and egg antibodies (triangles- NDV vaccinated females, circles- SalenvacT vaccinated females). Each point indicates the residual relationship between female antibody and a single egg at a certain point in time.



Using the actual calculated ratio of antibodies transferred I investigated the relationship between female immune responses, the proportion of antibodies transferred and other female traits (full model, Table 4.2). I found no relationship between the size of a female's own immune response and the proportion of antibodies transferred to her offspring, i.e. high responders didn't necessarily transfer the greatest percentage to their eggs. The ratio of antibodies transferred to eggs had no relationship with female's peak antibody titre ( $F_{1,14}=0.10$ ,  $p=0.751$ ), peak day ( $F_{1,14}=1.17$ ,  $p=0.298$ ), mean antibody titre ( $F_{1,14}=0.02$ ,  $p=0.893$ ) or pre-vaccination antibody level ( $F_{1,11}=2.59$ ,  $p=0.147$ ).

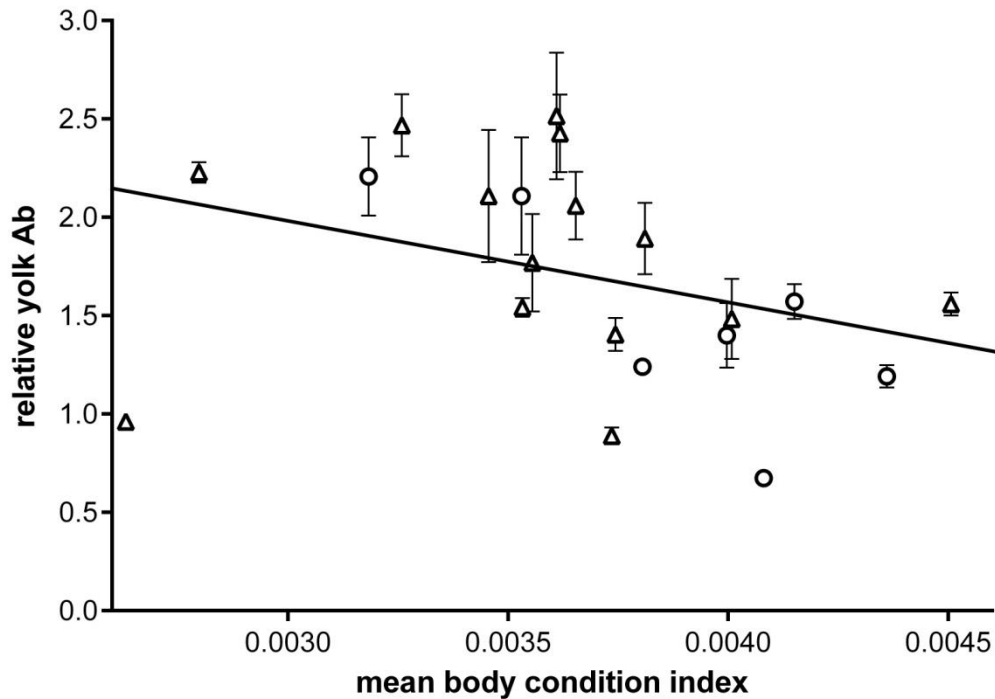
**Table 4.2** Maximal and Minimal linear mixed-effects model of the relative transfer of antibodies from mother to yolk, significant effects highlighted in bold.

RESPONSE	EXPLANATORY	Maximal Model			Minimal Model		
		df	F	p	df	F	p
Relative yolk Ab	Yolk mass	18	0.36	0.553	19	0.06	0.806
	<b>Female mean condition</b>	18	2.99	0.100	20	7.57	<b>0.021</b>
	Fat score	10	0.49	0.499	13	0.52	0.482
	Muscle score	10	0.001	0.998	10	0.00	0.998
	Egg investment	10	0.001	0.975	12	0.00	0.974
	Female start Ab	10	0.007	0.978	11	2.59	0.147
	Female peak Ab	10	0.42	0.528	14	0.10	0.751
	Treatment	10	1.55	0.240	15	0.08	0.775
	<b>Time (day)</b>	18	3.88	0.064	20	3.71	<b>&lt;.0001</b>
	<b>Interaction</b>						
	Female mean condition* Yolk mass	18	0.61	0.441	18	0.61	0.441

Random Effects	
	Variance component
Female ID	0.619
Residuals	0.335

I found no relationship between maternal condition and maternal circulating antibody levels or any of my other measures of antibody response (all  $p > 0.4$ ). However, in contrast, the relative amount transferred (ratio of maternal antibodies to egg antibodies) had a significant, negative relationship with mean body condition index at time of egg-laying;  $F_{1,20}=7.57$ ,  $p=0.021$ , where birds with high condition scores transferred a smaller ratio of antibodies than birds with lower condition scores (Figure 4.4). Female mean muscle and fat score for the experiment had no effect on the propensity to transfer antibodies to egg (muscle  $F_{2,10}=0.001$ ,  $p=0.998$ ; fat  $F_{2,13}=0.52$ ,  $p=0.482$ ; Table 4.2).

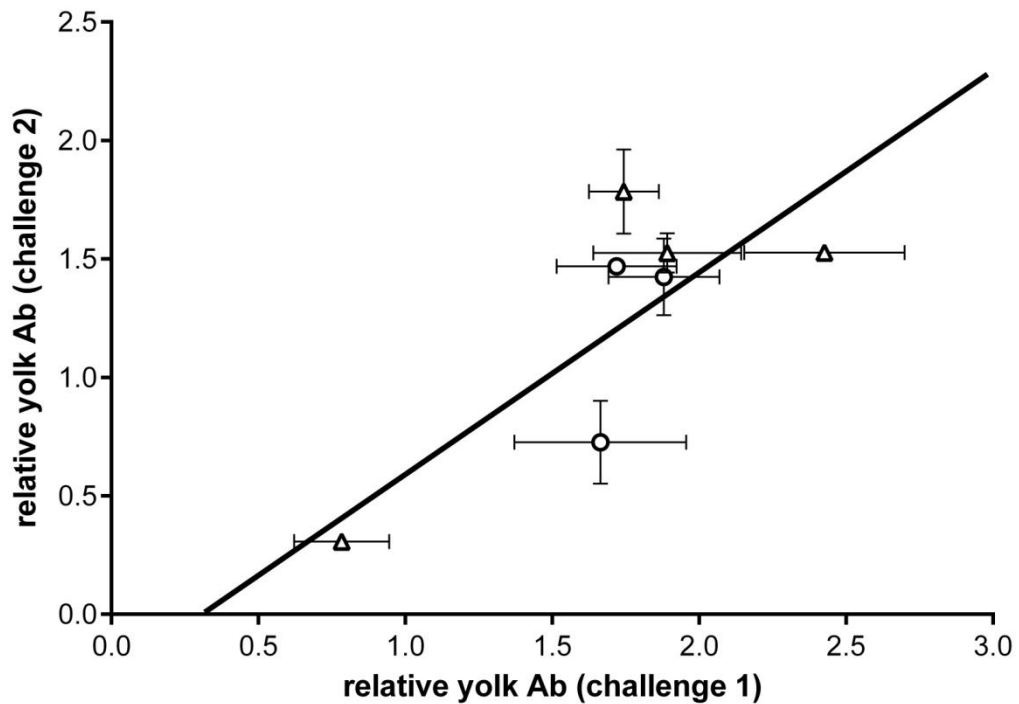


**Figure 4.4** Egg antibody level and female body condition. Relative transfer of female antibodies transferred to eggs in relation to female's mean body condition;  $F_{1,20} = 7.57$ ,  $P = 0.021$ ,  $n = 21$ .

I found no significant relationship between the amount of antibody transferred and total egg investment over the period of the experiment ( $F_{1,12} = 0.001$ ,  $p = 0.974$ ), suggesting that females who were transferring a greater ratio of their antibody level were not trading off costs associated with a higher transfer with resources allocated to reproduction in the same time frame. I also found no evidence for any trade-off between my other measures of a female's antibody response such as peak antibody or time to reach peak antibody with their total reproductive effort or female condition scores (body condition index, fat and muscle scores); all  $p$  values  $> 0.1$ .

A subset of females was challenged again at day 42 to look at the repeatability of transfer patterns across different challenge types: individuals that had been in the bacterial challenge group (Salenvac T group) were challenged with my viral NDV vaccine ( $n = 4$ ) and individuals from my viral challenge group (NDV group) were challenged with my bacterial vaccine (Salenvac T) ( $n = 3$ ). In these individuals I found a positive relationship between the relative level of antibodies transferred to eggs in response to the first challenge and the relative level transferred in response to the

second challenge, irrespective of which treatment was given first:  $F_{1,6} = 7.287$ ,  $p = 0.042$  (Figure 4.5). Again, using the log likelihood ratios (between models with and without female ID as a random effect), there was significantly more variation between individuals ( $X^2_1 = 6.07$ ,  $p = 0.01$ ) than within individuals and the estimate of across challenge repeatability ( $r = 0.577$ ) was again high.



**Figure 4.5** Positive correlation between mean ratio of antibodies transferred to eggs from females ( $n=7$ ) challenged twice. Circles represent females vaccinated with SalenvacT first, triangles represent females vaccinated with NDV first.

#### **4.4 Discussion**

Here I show that individual females vary in the timing and strength of their antibody production following an antigen challenge. However, most individuals are highly consistent in the ratio of antibodies transferred to eggs over the course of their immune response. Although individuals are consistent in the amount they transfer, some females consistently transfer more than others. Furthermore, this ratio was consistent across the two different challenges I administered, bacterial and viral, despite these challenges triggering different arms of the immune response. The ratio of circulating antibody that was transferred to the egg was independent of the strength of the maternal antibody response, suggesting different mechanisms determine these two traits. The amount of antibody transferred was negatively related to the female's body condition, but the female's circulating antibody level prior to transfer was not. I found no evidence for any trade off between levels of allocation of immunity to the egg or the female's circulating antibody level with overall levels of reproduction.

The results from this study demonstrate that a female's capacity to transfer antibodies to her eggs is not just related to her ability to produce an antibody response. This has important implications as it suggests that both a female's propensity to produce a particular level of immune challenge and her ability to transfer it to offspring may be two separate traits upon which selection might act. In natural populations, the fitness benefits of how protection is allocated between a mother investing in her own immune response and that of her offspring is therefore likely to depend on a number of factors including the conditions a mother finds herself breeding in and the likely pathogen exposure faced. Ultimately, any variation in the level of transfer will therefore create a more variable environment for pathogens and impact on any ensuing arms race between host and parasite.

A separation of the mechanisms responsible for the generation and transfer of an immune response is also potentially important commercially. Poultry lines have been established to select for genotypes that produce higher levels of antibody (Cheng and

Lamont, 1988, Parmentier et al., 2004). If the level of antibody transfer could further be selected upon this could potentially be advantageous. For example, the production of therapeutic and industrial antibodies in chicken eggs could be boosted, and the efficacy of maternal vaccination in agriculture enhanced.

This study also suggested that there may be consistency in antibody transfer across two vaccine types, suggesting generality of the trait. Between individual variation estimates and within-individual repeatability estimates for traits can be extremely useful in estimating the potential heritability of traits and their potential to evolve (Lynch and Walsh, 1998). However, to my knowledge there is very little information on between- and within-individual variability in levels of antibody transfer (or indeed any other immune components) in birds. The importance of establishing these values has already been highlighted by those investigating the role of other important egg constituents in avian development, such as maternal yolk testosterone. Similarly high estimates of repeatability of several key hormones have been found across breeding attempts (Gil et al., 2006, Tschirren et al., 2009) and between years (Eising et al., 2008) whereas other hormones appear to be regulated more by environmental variation (Tschirren et al., 2009). It is unclear whether females who transfer high levels of one maternal component such as antibodies may also transfer high levels of other key egg components in general. This would warrant further investigation to test if some females generally transfer more of all constituents or whether trade-offs occur (for example, more antibodies may be needed to balance hormonal immunomediated effects).

In this study the ratio of antibodies transferred to eggs had a significant negative relationship with body condition (an effect that was not found in Chapter 3). Other female condition-related traits such as fat and muscle scores were found to have no effect, though this may be due to the discrete scores having less discriminatory power than actual measures of female weight. The negative relationship between the antibody transfer and condition might appear counterintuitive, because it is often assumed that producing an antibody response is costly and that individuals in better condition can “afford” to invest more in transferring egg antibodies to their offspring

(Hargitai et al., 2006, Moreno et al., 2008, Pihlaja et al., 2006). However, a negative relationship between maternal condition and transfer has also been reported in a number of systems: Gasparini et al. 2007, for example, found a negative relationship between female's nutritional status and transfer in seabirds; non food-supplemented females transferred more resources, including antibodies, to offspring compared to food supplemented females. In Chapter 3, I found no relationship between condition and maternal antibody transfer; however, there was a relationship between condition and maternal antibody response, clutch size, and viability, suggesting a trade-off between immunity and reproduction. Additionally, due to the experimental design, any condition effects may have been masked due to an effect of first male traits on female condition. In *Daphnia* it is now well established that females experiencing low food availability produce offspring better able to prevent parasite establishment than high condition groups (Stjernman and Little, 2011). Landete-Castillejos et al. 2002 (Landete-Castillejos et al., 2002) found in Iberian red deer (*Cervus elaphus hispanicus*) that hinds from a low caloric-intake group had higher values of total antibodies in calves and produced less milk compared to calves of the control group, although this finding might arise due to poor quality females carrying a higher parasite burden. However, it has been hypothesised that this type of relationship could be a compensatory effect, with an individual of poor condition transferring more antibodies to offspring to compensate for their potentially higher susceptibility. In other studies the findings have been mixed; some studies in birds have found improving maternal condition can increase antibody transfer (Moreno et al., 2008), while others have found no effect of diet manipulation on immune transfer (Grindstaff et al., 2005). This relationship and how it may link to ecological differences between species, therefore warrants further investigation.

The non-passive transfer of antibodies to eggs could be explained by the condition-based mechanism, described above, or may simply result from differences between females that correlate with both their propensity to transfer antibodies and their mass to size ratio. For example, differences in condition may simply reflect differences in an individual's early conditions and their ability to compete and gain food which could in turn affect either the development of their receptor based system or their

ability to utilize this mechanism to maximize transfer. I examined whether the effect may arise from low condition females producing smaller eggs but transferring similar amounts of antibody with the result that yolk concentrations of antibodies could be higher. However, this did not appear to explain this result in this case; when yolk size was taken into account in my models, I found no interaction between maternal condition and yolk volume on the amount of antibody transferred. On other hand, female cumulative antibody level alone does not change with body condition scores, suggesting that the ability to produce an antibody response is not confined to the same factors, which determine propensity to transfer antibodies to eggs. I found no trade off between levels of allocation and levels of overall reproductive investment.

The mechanism underlying differences in the ability of females to transfer specific antibodies remain unclear. Transfer of maternal immunity in birds is a two-stage process. First, low levels of antibodies are transferred to the yolk as it develops and at oogenesis, then there is a sudden influx of antibodies and other yolk components in the last few days prior to egg formation in the reproductive tract (Kowalczyk et al., 1985). The transfer to the embryo from the yolk then mostly occurs in the few days prior to hatching by receptor-mediated transfer across the yolk membrane into the embryo's blood (Kowalczyk et al., 1985). The first part of this mechanism (from mother to yolk) is believed to be receptor mediated and individuals may differ in the number and effectiveness of this transfer process. However, the transfer of antibodies also appear to be directly correlated to the increase in size of yolk as an egg is formed (Kowalczyk et al., 1985), so investment may be tied to allocation of other constituents laid down in the last few days of egg formation. Eggs are produced on a 24-hour cycle so as one egg is laid, the yolk for the next day's egg has just been formed and packaged and is awaiting fertilization in the reproductive tract and the egg for the day after is still sequestering yolk constituents in the ovary. This sequential pattern of egg production over a series of days explains why eggs vary in their antibody level over the course of an immune response.

I have demonstrated that the propensity to transfer is consistent within individuals but varies between individuals. Could there also be adaptive allocation over and

above these effects? There is certainly evidence consistent with this hypothesis, with levels of antibodies varying with laying sequence (O'Brien and Dawson, 2009), brood value (Pihlaja et al., 2006) and sex of offspring (Martyka et al., 2011). However, my study does also highlight one variable that can affect the levels of transfer that often isn't accounted for in differential allocation studies. The single most important factor affecting how much antibody was found in the eggs was at what point in the immune response a female was at when a particular egg was laid. For example, if a clutch of eggs was collected from a female in the initial phase of her immune response – perhaps up to 10 days post-challenge – she would likely show a trend of laying eggs with higher levels of antibodies towards the end of the clutch, as antibody levels would be increasing on a daily basis and oocytes for successive eggs develop in 24 hour cycles and are laid approximately 24 hours apart. However, if I had collected a clutch of eggs slightly later, I might find females lay eggs with fewer antibodies in the end of the clutch as these are being formed at the time point just after the peak of her immune response, so subsequent eggs would sequester declining levels of antibodies from the female's circulating blood. Females show considerable variation in the magnitude and timing of peak responses, thus making it difficult to predict what an average response a challenge (or to natural levels of infection) might be. In natural systems, infection history may not be known and I have demonstrated that females can be very variable in both the magnitude and timing of their immune responses, even following a controlled experimental challenge; measuring maternal antibody levels while she is producing a clutch in these types of study would therefore be very informative. Furthermore, the discovery that the distribution of the sexes (Badyaev et al., 2002) and extra- pair paternity (EPP) offspring (Magrath et al., 2009) are often non-randomly distributed within a clutch demonstrates the need to ensure these correlated antibody levels with the point in a female's response are considered too. This does not necessarily mean that these patterns are not adaptive – directing particular offspring to a particular part of a clutch that may receive more or less antibodies may in itself be an adaptive strategy and a mechanism by which allocation could be adjusted. However, distinguishing between these alternatives is crucial in terms of our understanding of the mechanisms underlying these effects and their implications for offspring fitness as they develop.



# CHAPTER 5

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## **Discussion**

### **5.1 Maternal effects**

Maternally transferred substances are known to play a major role in offspring development and success. While past research has viewed them as a passive reflection of the maternal state more recent literature has viewed them as potentially flexible and adaptive mechanisms that females could employ to adjust the early environment of her offspring in ways that may increase Darwinian fitness (Mousseau and Fox, 1998a). While these studies explore patterns of allocation and their potential functionality, the mechanisms operating in the mother to transfer these substances remains unclear. Furthermore, the basic premise underlying many of these reported effects – that increases in allocation has an impact on current breeding success, future breeding success and/or maternal condition has seldom been addressed: As I have alluded to throughout the thesis, these mechanisms are highly important to a wide range of disciplines; here I discuss how this study has extended our knowledge and identified further questions in this field.

### **5.2 Differential allocation**

In Chapter 2 of this thesis, I explore differential allocation between clutches in response to mate switching, and how females may vary in their allocation capability under these conditions in a laboratory population of Chinese painted quail. The study highlighted original findings which make a contribution to my understanding of life history trade-offs and differential allocation theory. Clutch size was shown to vary in relation to the badge size of the male with whom a female was first paired for a given clutch. Furthermore, the probability of an egg being viable was strongly linked to

paternal badge size, suggesting some inherent benefit of mating with a large badge male. Due to using a cross-over design and allowing females to lay four clutches, this finding provides clear evidence for differential allocation in relation to clutch size and a relationship between the male trait on which this allocation is based on embryo viability. Thus, female Chinese painted quail possess an intrinsic ability to adjust their clutch size with regard to partner traits. The experimental design implemented also highlighted an important finding that early reproductive allocation patterns can persist to influence offspring in future breeding attempts later in a female's life. Notably, it was determined that egg mass was significantly related to the male with whom a female was initially paired with at the beginning of the experiment, illustrating that a female's previous experience can carry-over into future reproduction. Untangling this carry-over effect on offspring viability from other factors related to offspring traits would not have been possible to see if I had only explored one or two clutches per female. This carry over effect also had a detectable effect on embryonic development with embryos being significantly heavier at day 3 (approximately 1/5th of the way through their total development time) if females had initially been paired to large badged males for their first breeding attempt. To my knowledge this is the first study to demonstrate that maternal allocation decisions may affect embryo growth.

This experiment has demonstrated several key assumptions of differential allocation theory (Rutstein et al., 2004) i.e. that females alter investment strategies based on male signals, that allocation is varied by the same female across breeding attempts with different males, that allocation decisions impact positively on offspring traits. However, one aspect I couldn't test directly was the potential impact of these allocation decisions on female fitness – the nature of my experiment meant that because a balanced design was used to look at the effects of different males on allocation – all females had equal opportunities to breed with large and small badged males across their reproductive lifespan during the experiment. An interesting future experiments would be to alter allocation from the second clutch onwards in a consistent direction to look at the consequences of increasing investment for preferred males on female fitness – monitoring egg number and female survival

would allow both the cost of the increased egg production on survival and the potential gain in offspring produced to be calculated and the net benefit of differential allocation on female fitness to be measured. It would also be interesting to repeat this experiment and extend it until all females died naturally to measure the impact of initial allocation decisions and their carry-over effect on offspring quantity and quality and female survival. I would also have liked to be able to examine what components of the egg are altered when egg size is changed in response to male traits. Do all egg components increase proportionately or are yolk versus albumen constituents altered more – both yolk and albumen are known to be important for embryonic growth (Bonisoli-Alquati et al., 2007) so either may be accounting for the observed effects on embryo growth rate in this study.

### **5.3 Maternal allocation of antibodies**

In Chapter 3 of this thesis I investigate factors affecting the transfer of maternal antibodies to eggs. Several important findings were found. First, I show the major influx of antibody disposition starts approximately 5 days prior to the egg being fertilised and 6 days before it is laid however, it is only 1 and 2 days before fertilisation of an egg that the ova shows a significant correlation with female antibody levels. This suggests egg components are not all laid down in proportion to each other at this developmental stage.

Male traits did not appear to affect antibody concentrations in the egg. However, females did produce more eggs when paired to large badged males so they may have had to up-regulate antibody production to supply eggs with antibodies for longer. However, I also demonstrate a potential cost associated with antibody responses in that females with a higher antibody response produced fewer viable embryos. No variation in the level of maternal antibodies was found for eggs of male and female offspring at the embryo stage at pre- or post- incubation stages. Moreover, there was no difference in the relative level found between sexes (when accounting for female levels). Other studies exploring maternally transferred antibodies and embryo sex have only explored antibody levels post incubation (Martyka et al., 2011, Saino et al., 2003).

Changes in provisioning of egg mass appeared at first to have no effect on the levels of antibody transferred to the egg. However, calculations of total antibody level were based on yolk size; yolk size could only be measured at 72hrs incubation and therefore it is unclear how much yolk and albumen had been used in embryo growth. In Chapter 2 females initially paired to large badge males produce eggs that have larger embryos at 72hrs compared to females initially paired with small badge. This would suggest that there has already been differential use of egg components at this stage. If I calculate total antibody level based on egg mass then the effect of initial male is significant. We therefore cannot discount that a carry-over effect is impacting on total antibody levels. An obvious extension of this work would be to test this directly by exploring the effect the initial male pairing has not only on external egg traits but the level of different egg components such as yolk and albumen ( and the composition of these) at pre-incubation stages.

The novel technique implemented here was highly important for determining potential sex difference between eggs in antibody titres pre and post incubation. However this may have caused a decrease in the number of viable eggs. This is a constraint of the results in Chapter 3, as having a larger sample size would have allowed us to test this more robustly and to be more confident in the non-significant effects found. The technique implemented in this chapter is important for exploring allocation of maternal antibodies to yolks pre incubation, and could be highly useful for exploring other maternally transferred yolk components. Despite this problem the technique is effective, and therefore these findings, along with the method implemented, are highly useful in increasing our knowledge of maternal effects.

#### **5.4      *Repeatability of maternal antibody transfer***

In Chapter 4 of this thesis, I describe the findings of a laboratory study exploring allocation in continually laying females to allow antibody levels in both mother and egg to be tracked over time following a series of different types of immune challenge. The results show that there is substantial variation between females in the proportion of their own antibodies they transfer to their offspring. Furthermore, while

this transfer varies between females it does not covary with the height of the mothers own immune response. The only female trait that appears to influence transfer was female condition index, where females of a lower condition index transferred relatively more antibodies compared to females of a higher condition index. Finally, and most importantly, the repeatability for an individual female in her transfer of antibodies was not only robust across her immune response to one challenge, but robust across challenge types. This suggests that maternal antibody transfer may be determined by a mechanism which is not specific to one type of antibody.

Domestic animal industries often produce high numbers of animals in relatively small areas therefore there is a large interest into control of disease. Maternal antibodies can be highly beneficial in the farming of poultry and cattle but can also be detrimental to vaccination protocols. In the poultry industry in particular female hens are often vaccinated for particular pathogens prior to laying eggs to increase the likelihood of that antibody protection being transferred to chicks. However both in poultry and cattle vaccination protocol for newborns often has to be timed particularly carefully to make sure the vaccine is effective and not being “blocked” by maternal antibodies present (for example with Marek’s disease). Therefore furthering our understanding of maternal antibodies in the quantity and timing of transfer is useful to a wide range of disciplines. Within the poultry industry there is a key interest in selecting animals with high immune responses (Cheng and Lamont, 1988, Parmentier et al., 2004). However to my knowledge it is unclear if these individuals are also high transfers of antibodies or if this increase ability to produce an immune response has a negative impact on their offspring’s viability. Therefore the findings in this thesis regarding the transfer of maternal antibodies and their potential effect offspring is potential important to industry. Whatever the aim, knowledge of these mechanisms would allow more informed decisions to be made to increase the production, health and welfare in animal production.

## **5.5 Concluding remarks**

Individuals are not homogenous in their provisioning of offspring, even when they are kept under the same conditions. There have been large steps forward into understanding the transfer of maternal antibodies, and equally large advances in the

literature regarding differential allocation theory. However in both topics there are key areas which are still understudied. This study addresses some of the gaps in the literature, and reports some novel findings that should give direction to further research.

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# Appendix 1

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## Exploratory behaviour (Chapter 2)

### A.1.1 Behavioural traits and maternal allocation

Maternal condition has been linked to various personality traits, therefore personality traits could relate to allocation decisions when paired to attractive or unattractive partners. An individual's behavioural traits in a wild environment could give them a better chance of getting resources or chance of survival against predators, therefore they can have fitness consequences (Adriaenssens and Johnsson, 2013) and could covary with life-history traits (for review see, Réale et al., 2010). A behaviour which can give an individual a lot of information about their environment is exploratory behaviour; in studies this behaviour has been found to be repeatable within individuals (Dingemanse et al., 2002) and laboratory studies of this behaviour have been found to correlated with types of movement in the wild (Herborn et al., 2010). However, the relationship between laboratory tests of exploration and wild equivalents may not always be correlated and may be context specific (Minderman et al., 2010). Despite these inconsistencies in the research between wild and laboratory studies, exploratory behaviour and fast exploration has been shown in male great tits to include individuals likely to be of a high aggression, whereas, slow explorers have been shown to be relatively non-aggressive and shy (Verbeek et al., 1996, Verbeek et al., 1999). These sorts of behavioural traits have been linked to life history traits in a number of studies. Although there is not a large amount of evidence directly related to DA theory, a number of studies explored aggression and exploratory behaviour in females and males and found them to impact on reproduction (for review see, Smith and Blumstein, 2008). Experiments exploring female personality traits have had mixed findings with regard to clutch traits; with no relationship on clutch size or egg mass but a positive relationship between female dominance and testosterone levels in yolks (Tanvez et al., 2008). Comparatively, nest success, fledging size and condition

have been found to relate to exploratory behaviour (Both et al., 2005) and the number of offspring fledged has also been related to exploratory behaviour of mothers (Dingemanse et al., 2004). We aim to account for female traits which are often neglected when exploring differential allocation, to determine if all females respond in the same way in relation to differential allocation or if traits related to a females condition or behaviour cause variation in this allocation.

### **A.1.2 Behavioural traits methodology**

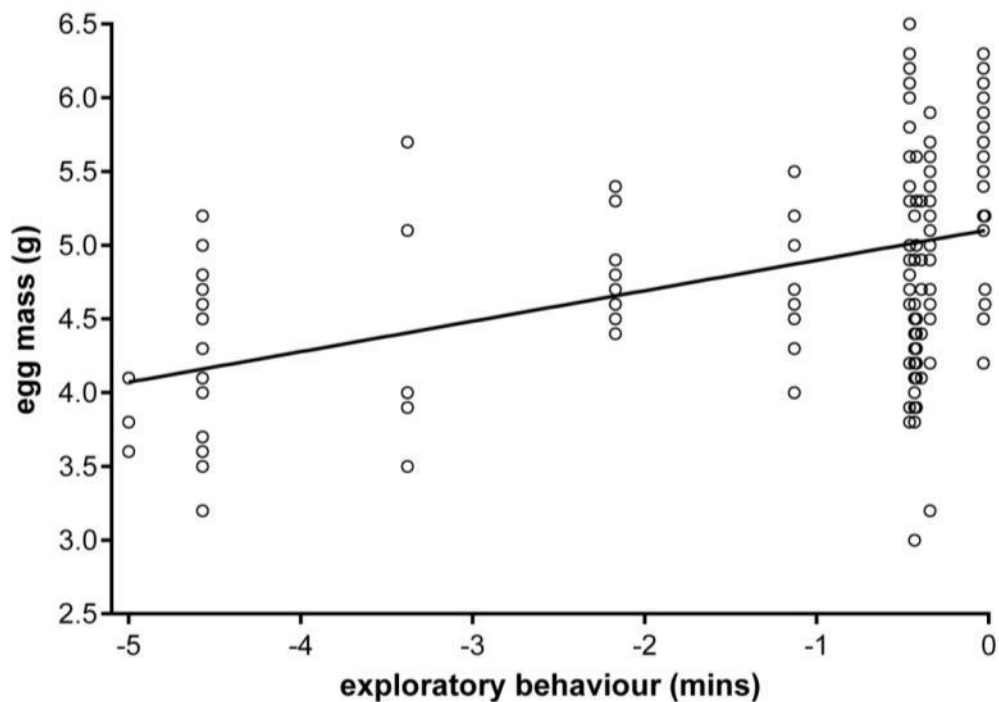
Exploratory behaviour is a commonly measured trait to assess boldness, This was determined for each individual male and female by measuring the amount of time it took for an individual to emerge from a box and enter a “novel” environment. Personality tests were conducted outwith the home cage of the focal bird. An experimental cage was established (800 x 500 x 375 mm) similar to the home cage (food and water was also provided). The cage included a box filled with wood chip with a door through which a test bird could enter the novel environment. Each bird was individually removed from their home cage and placed directly into the box. After two minutes the observer opened the door of the box, stepped behind screen, and began timing (the focal bird was observed via a small gap in the screen). The time taken for the individual to leave the box was recorded and if it took more than 5 minutes for the individual to appear from the box the individual was marked as > 5 minutes (6 male, 3 female). Repeatability of this behavioural trait was determined using three recordings *per* individual of time to enter a novel environment (randomly allocated order), the intraclass correlation coefficient for these measures was  $r = 0.754$  ( $n = 44$  individuals). Rank order of boldness was highly consistent between the three measurement periods;  $r_s = 0.67$ ,  $p < .0001$  (Spearman's  $\rho$  correlation).

### **A.1.3 Behavioural traits results**

There was a significant correlation between male exploratory behaviour and badge area;  $r = 0.288$ ,  $d.f = 19$ ,  $p = 0.036$ , however male exploratory behaviour was never retained in any models.

Female exploratory behaviour was positively related to egg mass;  $t = 2.25$ ,  $p = 0.0289$  but this relationship did not show any relationship with male traits. Females who spent a shorter time leaving the box had eggs of a greater egg mass (Figure A.1), suggesting that this personality score (exploratory behaviour) may reflect some underlying condition of a female, although no correlation between initial female condition index and personality score found;  $r = 0.041$ ,  $p = 0.507$  (Pearson's correlation).

Exploratory behaviour had no significant relationship with egg viability;  $z = -0.62$ ,  $p = 0.531$ . There was also no relationship between exploratory behaviour and mortality in the experiment;  $t = 0.62$ ,  $p = 0.534$ .



**Figure A.1** Individual egg mass per egg laid in relation to female exploratory behaviour (time to enter novel environment). Regression line indicates positive relationship;  $t = 2.25$ ,  $p = 0.0289$ .

#### **A.1.4 Behavioural Traits Discussion**

##### ***A.1.4.1 Male exploratory behaviour***

Although there was evidence for female allocation via cues from male badge size, and male badge size correlated with male exploratory behaviour, there was no relationship between reproductive allocation and male personality, which may be due to the type of personality trait measures. In a recent meta-analysis (Smith and Blumstein, 2008) the authors found that behavioural traits related to boldness across species appeared to co-vary with reproductive success (in particular males), whereas exploratory behaviour appears to be more linked to survival. However it has been documented in many species that dominance and plumage have a positive relationship (for review see, Santos et al., 2011), therefore any relationship found may have co-varied with the findings for male badge size. Therefore the lack of effect of male exploratory behaviour may be due to using this trait and a dominance trial would be useful to determine if this personality trait influenced females along with the size of badge a male has.

##### ***A.1.4.2 Female exploratory behaviour***

There appears to be a relationship with the female personality trait that was recorded (exploratory behaviour) and egg size. Females who were faster to explore a novel environment produced larger eggs than females who were slower (in clutch 1 and when not accounting for initial male carry over effect), suggesting that the personality score collected may represent a female trait linked to reproduction. This type of finding is not as well documented in the literature, and the studies there are show mixed results; no relationship between maternal social hierarchy with clutch size or egg mass (Tanvez et al., 2008), compared to positive relationship between exploratory behaviour and fledging success (Both et al., 2005).

